Fajon belüli adaptív változatosság: vizsgálatok a fenotípustól a genotípusig

Doktori értekezés

Magyar Tudományos Akadémia

Herczeg Gábor

Eötvös Loránd Tudományegyetem

Állatrendszertani és Ökológiai Tanszék

Budapest

2014

dc_892_14

Tartalomjegyzék

1. Bevezetés 1
1.1. A fenotípusos változatosság mögötti mechanizmusok 1
1.2. A fenotipikus plaszticitás és variabilitása 3
1.3. A fenotípusos változatosság kvantitatív genetikai háttere 4
2. Célkitűzések 6
3. Anyag és módszer 8
3.1 A vizsgált faj 8
3.2 A vizsgálati terület 11
3.3 A halak gyűjtése, konzerválása 12
3.4 Fogságban tartás, szaporítás és kezelések 12
3.5 A főbb vizsgált változók 14
3.5.1 Morfológia 14
3.5.2 Életmenet 15
3.5.3 Idegrendszer, érzékszervek 16
3.5.4 Viselkedés 18
3.6 Genetikai vizsgálatok 18
3.7 Statisztikai analízisek 19
4. Eredmények és megvitatásuk 19
4.1 Populációs különbségek 20
4.1.1 Populációgenetikai elővizsgálat 20
4.1.2 Morfológiai különbségek 23
4.1.3 Életmenetbeli eltérések 26
4.1.3.1 Testméret, növekedés, ivarérés időzítése 26
4.1.3.2 Ivari méretdimorfizmus és fekunditás 33
4.1.3.3 Energiaraktárak 36
4.1.4. Agy és érzékszervek 37
4.1.4.1 Agyméret és az agyterületek mérete 37
4.1.4.2 Oldalvonalszerv 42
4.1.4.3 Színlátás 45
4.1.5 Viselkedés 49
4.1.6 Fejlődési stabilitás 50
4.1.7 A természetes szelekció szerepének tesztelése 53
4.1.8 Összegzés 55
4.2 Fenotípusos plaszticitás 57
4.2.1 Ragadozó jelenlét/hiány és a táplálék mennyisége 57
4.2.1.1 Morfológia 59
4.2.1.2 Életmenet 61
4.2.1.2.1 Testméret 62
4.2.1.2.2 Energiaraktárak 64
4.2.1.3 Agy és érzékszervek 67
4.2.1.3.1 Agyméret és az agyterületek mérete 67

4.2.1.3.2 Oldalvonalszerv 69
4.2.1.4 Viselkedés 70
4.2.2 Társas élet hatásai 72
4.2.2.1 Testméret 73
4.2.2.2 Agyméret és az agyterületek mérete 75
4.2.3 Összegzés 76
4.3 Kvantitatív genetikai háttér 77
4.3.1 A populációs szétválás háttere 77
4.3.1.1 Testméret 79
4.3.1.2 Ivarérés időzítése 81
4.3.1.3 Táplálkozási aktivitás 82
4.3.2 Szelekció alatt álló genom-régiók 85
4.3.2.1 *Genome scan* 85
4.3.2.2 *QTL mapping* 86
4.3.2.2.1 A hasi tüske és a mell-öv redukciójának genetikai háttere 87
4.3.2.2.2 A testméret és a növekedési ráta genetikai háttere 91
4.3.2.2.3 A viselkedés genetikai háttere 94
4.3.3 Összegzés 96
5. Összefoglalás és kitekintés 96
6. Köszönetnyilvánítás 98
7. Az értekezés alapjául szolgáló saját közlemények listája 101
8. Irodalomjegyzék 105

9. Függelék

dc_892_14

dc_892_14

1. Bevezetés

Az evolúciós ökológia fő tárgya a természetben megfigyelt fenotípusos változatosság mögötti okok és mechanizmusok feltárása. Az evolúció (egy szaporodó közösség genetikai állományának generációk közötti változása) sokrétű folyamatai közül a fenotípust is érintő változások kis arányt képviselnek, és ez az arány még kisebb, ha csak az adaptív változásokra gondolunk. Ennek dacára, az adaptív evolúció vizsgálata a legnépszerűbb, és talán legfontosabb szelete a tudományterületnek.

Evolúciós adaptációt több szinten lehet vizsgálni. Gyakoriak a fajok vagy magasabb taxonómiai szintek közötti, vagy éppen ellenkezőleg, a populáción belüli, egyedek közötti változatosságot célzó kutatások. E két megközelítési szint között találjuk a fajon belüli, populációk közötti összehasonlításokat, melyek hathatósan segíthetik a konkrét környezeti változók által generált evolúciós változások feltérképezését. Egy adott fenotípusos tulajdonság adaptív evolúciójának megértéséhez általában három dolgot kell vizsgálni: az egyedek közötti változatosságot, az egyedek közötti változatosság genetikai hátterét és a különböző fenotípusú egyedekre ható szelekciós erőket. Noha egy-egy kiemelt ökológiai fontosságú tulajdonság vizsgálatával sok mindent megtudhatunk az adott modell-rendszer evolúciójáról, a kurrens fenotípusos integrációs elméletek fényében a rendszer valódi értelmezéséhez a lehető legtöbb releváns tulajdonság követésére van szükség.

Disszertációmban a fajon belüli fenotípusos változatosság mögötti mechanizmusokat igyekszem felderíteni, egy rendkívül variábilis hal, a kistestű kilenctüskés pikó (*Pungitius pungitius*) szélsőségesen eltérő környezetekhez alkalmazkodott populációinak vizsgálatán keresztül. A vizsgálatot több, egymásra épülő szinten végeztem. A tulajdonságok „egyszerű” populációs összehasonlításától kiindulva tárgyalom a fenotipikus plaszticitás eltéréseit populációk és tulajdonságok között, valamint a populációs különbségek kvantitatív genetikai hátterét, majd végül kísérletet teszek a tulajdonságok változatossága mögött álló genomikai régiók azonosítására. A vizsgált tulajdonságok tekintetében is igyekeztem a lehető legszélesebb merítést tenni, morfológiai, életmenet, neurobiológiai és viselkedési karaktereket egyaránt górcső alá véve.

1.1. A fenotípusos változatosság mögötti mechanizmusok

A környezet térbeli és időbeli heterogenitása a szelekciós erők heterogenitását okozza, ami gyakran felelős a fajok populációi között megfigyelt fenotípusos és genetikai szétválásokért (Mayr 1963; Endler 1977). A helyi környezeti sajátosságokhoz való – genetikai változatokon alapuló – alkalmazkodást nevezzük lokális adaptációnak, és mivel mind a természetes szelekció, mind a legtöbb ökológiailag releváns tulajdonság heritabilitása általánosan megfigyelt jelenség (Houle 1992; Kingsolver et al. 2001), a megfigyelt fenotípusos változatosságot leginkább a lokális adaptációkkal magyarázzák (Schluter 2000; Merilä & Crnokrak

2001; Leinonen et al. 2008). Ennek dacára, a populációk közötti genetikailag meghatározott fenotípusos szétválásokra más magyarázat is van, például a tulajdonságok különbségét okozhatja a random genetikai sodródás is (Lande 1976). A természetes szelekció és a random genetikai sodródás hatásainak szétválasztására nagyszámú populáció vizsgálatba vonása és a megfelelő neutrális genetikai információ ismerete esetén megvan a módszertár (Merilä & Crnokrak 2001; Leinonen et al. 2008; 2013). Alacsonyabb számú populáció vizsgálatakor, és/vagy genetikai információ hiányában azonban ezek a módszerek nem alkalmazhatóak (O'Hara & Merilä 2005). Ilyen estekben egyszerűen a populációk közötti eltérések szisztematikusságát, azaz környezet (habitat)-függését vehetjük a természetes szelekció indikátorának (Clarke 1975; Endler 1986; Schluter & Nagel 1995; McGuigan et al. 2005).

A környezeti változatossághoz való alkalmazkodásnak a lokális adaptáción kívül van egy másik, szintén drasztikus fenotípusos különbségeket okozó mechanizmusa, a fenotipikus plaszticitás (egy genotípusból eltérő fenotípusok fejlesztése, West-Eberhard 2003). Érdekes módon előfordulhat, hogy a generációkon keresztül, genetikai szinten működő lokális adaptáció és az egyedfejlődés szintjén operáló fenotipikus plaszticitás egymással ellentétes irányba hat, aminek eredményeképp a természetben nem figyelhető meg változatosság (*countergradient variation*; Conover & Schultz 1995). Mindezek függvényében a természetben megfigyelt fenotípusok alapján levont evolúciós következtetések – mivel a fenotipikus plaszticitás nem zárható ki – elhamarkodottnak tekinthetők, és gyakran hibásak (Kuparinen & Merilä 2007; Merilä 2009). Persze ez nem jelenti azt, hogy a fenotipikus plaszticitásra, mint zajra, kiküszöbölendő zavaró tényezőre kellene tekintenünk (Falconer 1952; Pfennig et al. 2010), hiszen önmagában is jelentős rátermettség-növelő hatással bír (Gomulkiewich & Kirkpatrick 1992; Ghalambor et al. 2007; Beldade et al. 2011), sőt, a modern evolúciós szemlélet szerint a lokális adaptációhoz szükséges első lépesként is felfoghatjuk (West-Eberhard 2005; Pigliucci et al. 2006; Pfennig et al. 2010).

Bárhogy is tekintünk a fenotipikus plaszticitásra, a korrekt evolúciós interpretáció érdekében szükség van a lokális adaptáció és a fenotipikus plaszticitás szerepeinek tisztázására a természetben megfigyelt adaptív változatosság létrehozásában, vagy legalábbis a lokális adaptáció fenotipikus plaszticitástól mentes becslésére. Erre a két fő módszer a *common garden* és a *reciprocal transplant* (talán a „közös környezet" és „kölcsönös áthelyezés" lehetne a magyar megfelelőjük, de mivel tudommal magyar kifejezések nincsenek használatban, a továbbiakban a közismert angol kifejezéseket használom) kísérleti elrendezések alkalmazása (Spicer & Gaston 1999; Kawecki & Ebert 2004). Az első módszernél a különböző populációkból származó egyedeket egy standardizált, közös környezetben neveljük fel, mintegy kiiktatva a környezeti változatosság által direkt módon indukált plaszticitást. Így a kísérletben megfigyelt változatosság már nagy valószínűséggel genetikai hátterű (a biztos eredményekhez több laborgeneráció, vagy komplex

keresztezések kellenek; Lynch & Walsh 1998). A második módszer lényege, hogy a különböző populációkból származó egyedeket mind a saját, mind a többi vizsgált populáció természetes élőhelyein neveljük. Ezen a módon a lokális adaptáció és a fenotipikus plaszticitás hatásait közvetlenül becsülhetjük. Az utóbbi módszer persze rendkívül munkaigényes és logisztikailag/etikailag is problémás, ezért a környezeti variabilitást is gyakran inkább egy *common garden* kísérlet keretein belül próbáljuk rekonstruálni. Amennyiben a kísérlet eltérő környezetekben (akár a természetben, akár manipuláció eredményeként laborban) zajlik, a kétféle (genetikai és környezeti) komponens elkülönítésén kívül a kettő interakcióját, azaz a genetika-függő környezet indukálta választ is tesztelhetjük. Megfelelő keresztezési elrendezéssel (pl. több hím több nősténnyel párosítva, vagy populációk közötti hibridek létrehozása) vagy a természetes populációkban sok-generációs pedigrék mentén mért tulajdonságok felhasználásával még mélyebbre áshatunk, és becsülhetjük a főbb kvantitatív genetikai paramétereket, mint például az additív genetikai hatást, nem-additív genetikai hatásokat (pl. dominancia), környezeti hatást és az anyai hatást (Wright 1978; Falconer & McKay 1996; Lynch & Walsh 1998).

1.2. A fenotipikus plaszticitás és variabilitása

A fenotipikus plaszticitás meglehetősen nagy tudományos publicitást kapott az elmúlt években. Sokáig csak, mint a lokális adaptációs mintázatokat elfedő zajt kezelték az evolúcióbiológusok (Falconer 1952), de mára egyértelművé vált a jelenség biológiai szignifikanciája (Pfennig et al. 2010). Gyakorlatilag az evolúcióbiológia egyik központi kérdésévé nőtte ki magát, összekötvén a genetikát a fejlődésbiológiával. Ennek dacára, még nincs teljesen egyértelmű konszenzus a fenotipikus plaszticitás jellegéről, egyesek szerint ez is egy ugyanolyan összetett kvantitatív tulajdonság, mint például a növekedési ráta (de Jong 2005), míg mások szerint ez egy olyan fejlődési folyamat, ami elősegíti az adaptív szétválást és végső soron a fajképződést (Pigliucci et al. 2006; Crispo 2007; Pfennig et al. 2010). Jelenleg a második nézet az uralkodó. Mindenesetre a fenotipikus plaszticitás evolúciója, azaz a polifenizmus – polimorfizmus átmenet bármelyik irányban egy izgalmas téma, amit éppen csak elkezdtünk értelmezni (Fusco & Minelli 2010).

Egy egyed sok szinten és módon mutathat fenotipikus plaszticitást. A környezet változására a génexpresszió regulációjától a morfológiai struktúrák szélsőséges módosításáig terjedő skálán képzelhető el plasztikus válasz, amely, gyakran az érintett tulajdonságtól függően lehet irreverzibilis, flexibilis vagy reverzibilis, illetve megjelenhet jól elkülönülő állapotok formájában vagy egy folyamatos tulajdonság-grádiens mentén (West-Eberhard 2003; Crispo 2008; Whitman & Agrawal 2009). A válasz esetenként csak egy fejlődési stádiumban figyelhető meg, de előfordul a teljes élet alatti, vagy akár – anyai hatásokon keresztül – generációkon átívelő hatás is (West-Eberhard 2003).

Jogosan merül fel a kérdés, hogy milyen körülmények között jelenik meg a fenotipikus plaszticitás képessége? Nyilvánvalóan ez a plaszticitás helyi költség-

nyereség viszonyaitól függ (DeWitt et al. 1998). Általában az időszakosan vagy finom térskálán változó környezet, a változások prediktálhatósága, és a lokális adaptáció ellen ható erős génáramlás teszi a fenotipikus plaszticitást előnyössé (Via 1995, Sultan & Spencer 2002; West-Eberhard 2003; Crispo 2008). Térben és időben stabil környezetben a fenotipikus plaszticitás eltűnhet a genetikai asszimiláció (egy adott fenotípus kanalizálódik, és már környezeti inger nélkül is kifejeződik) következményeként, vagy eltűnhet még a környezet stabilitásától függetlenül a random genetikai sodródás és káros mutációk eredményeként is (Waddington 1953, Crispo 2007, 2008; Masel et al. 2007).

A fenotipikus plaszticitás evolúciójának megértése az evolúcióbiológia egyik legégetőbb kérdése (Pigliucci 2005). Az egyik legkézenfekvőbb megközelítés lehet a különböző környezethez adaptálódott populációk összehasonlítása, azaz a fenotipikus plaszticitás fajon belüli variabilitásának vizsgálata. Az abiotikus (pl. kiszáradás esélye kis tavaknál, anoxia) és biotikus (pl. predációs nyomás) környezeti hatások tekintetében eltérő populációk képességét a fenotipikus plaszticitásra többen is vizsgálták eltérő eredményekre jutva a populációs különbségek és a fenotipikus plaszticitás mértékének összefüggése tekintetében (De Meester 1993; Laurila et al. 2002; Van Buskirk and Arioli 2005, Crispo & Chapman 2010a,b). Az ilyen típusú vizsgálatok száma azonban még alacsony, és az egyes vizsgálatok általában behatároltak a vizsgált tulajdonságokat illetően. Ezért szükség van olyan kutatásokra, ahol az eltérő környezethez lokálisan adaptálódott populációk képességét az adaptációt kiváltó környezeti faktorok által indukált fenotipikus plaszticitásra a lehető legtöbb releváns tulajdonság bevonásával tesztelik.

1.3. A fenotípusos változatosság kvantitatív genetikai háttere

A természetben megfigyelt – akár egyedek, akár populációk közötti – fenotípusos változatosság vizsgálatánál felmerül az igény, hogy a változatosság létrehozásáért potenciálisan felelős komponensek (pl. additív genetikai hatás, dominancia, anyai hatás, környezeti hatás) szerepét számszerűsítsük. Erre már jó ideje számtalan kísérleti és matematikai módszer áll rendelkezésünkre (Falconer & McKay 1996; Lynch & Walsh 1998). A vizsgálatok legnagyobb része a populáción belüli kérdésekkel (heritabilitás, tulajdonságok közötti genetikai korrelációk) foglalkozik (pl.: Leinonen et al. 2010, 2011). Ehhez képest talán kissé háttérbe szorultak a populációk közötti fenotípusos különbségek megértését célzó vizsgálatok, pedig a populációk közötti hibridek vizsgálatán alapuló módszertan már régóta ismert (Wright 1978). Persze az izgalmas mintázatokat mutató fajok egy részénél ezek a módszerek csak nagy nehézségek árán, vagy egyáltalán nem kivitelezhetőek, de még így is marad elegendő modell. Például Laugen et al. (2002) gyepi békáknál (*Rana temporaria*) vizsgálta a szélességi övek mentén megfigyelt lárvakori rátermettséget becslő változók (növekedési ráta, kor és méret a metamorfóziskor) populációk közötti változatosságának hátterét. Két egymástól 1000km távolságra lévő populáción alapuló egy generációs reciprok keresztezési sémával sikerült megerősíteniük az additív és nem-additív genetikai, valamint az anyai hatások

jelenlétét. Még több lehetőség rejlik olyan fajok vizsgálatában, ahol több laborgeneráció is aránylag könnyen létrehozható. Huttunen & Aspi (2003) a *Drosophila virilis* muslica faj két laboratóriumi törzse között megfigyelt párzási viselkedésbeli eltérés hátterére volt kíváncsi. Két hibridgeneráció vizsgálatával (a második generációban már 12 különböző hibrid vonal volt) már a lehetséges hatások közötti interakciókat, például az Y kromoszóma és egyéb faktorok, vagy a permanens citoplazma faktorok és egyéb faktorok kölcsönhatásait is tesztelni tudták. Az ilyen jellegű vizsgálatok száma azonban alacsony, pedig akár már egy hibridgeneráció vizsgálatával is értékes eredményeket kaphatunk olyan esetekben, amikor a habitat-függő populációs szétválás ökológiailag releváns tulajdonságoknál bizonyított, ám a direkt környezeti hatás nem zárható ki és ezért az evolúciós interpretáció gyenge lábakon áll.

A kvantitatív genetika végső célja a fenotípusos változatosság konkrét genetikai hátterének feltárása, azaz a változatosság kialakításában részt vevő gének azonosítása, és a hozzájuk kapcsolódó hatásmechanizmus leírása. Az alkalmazható genomikai módszerek száma magas és napjainkban robbanásszerűen növekszik, de természetes populációkkal kapcsolatban alapvetően két megközelítésről beszélhetünk. Az egyik módszer (*genome scan* vagy *hitchhiking mapping*, magyarul talán a „genom szkennelés" lehetne a fordítás, de a továbbiakban az angol kifejezést használom) rögtön a genommal kezd: a különböző populációkból származó egyedek genomjait nagyszámú feltételezetten neutrális markerrel 'szkennelik', és aztán éppen a neutralitástól való eltérés alapján azonosítják a stabilizáló vagy szétválasztó szelekció alatt álló genom szakaszokat, melyek szekvenálásával már a konkrét gének is azonosíthatók (Schlötterer 2003; Storz 2005; Vasemägi and Primmer 2005). Ennek egy fejlettebb változatánál nem feltételezetten neutrális markereket használnak, hanem a markereket az *a priori* kiválasztott gének közelébe vagy a nem kódoló szakaszaiba tervezik, és így közvetlen módon tesztelhetik az adott gének szerepét a populációs szétválásban (Shikano et al. 2010a,b). Ezeket a módszereket általában egyértelműen eltérő környezetben élő populációknál használják ahol pedigré, és a pedigréhez rendelhető fenotípusos adatbázis nem áll rendelkezésre. A másik módszer (*Quantitative Trait Locus [QTL] mapping*, talán a Kvantitatív Tulajdonság Lókusz Térképezés lehetne a fordítás, de a továbbiakban a *QTL mapping* kifejezést fogom használni) viszont éppen a pedigréhez rendelhető fenotípusos adatbázisra épül (Erickson et al. 2004; Slate 2005). Itt is az előző módszernél ismertetett marker típusokat használva 'szkennelik' a genomot, majd a fenotípusos és genetikai változatosság korrelációi alapján direkt kapcsolatokat keresnek adott tulajdonságok és genomrégiók között.

Mindkét módszerrel számos értékes eredmény született. A *genome scan* módszerrel Shikano et al. (2010b) képes volt konkrét géneket azonosítani egy halfaj tengervíz – édesvíz adaptációjában. A *QTL mapping* alkalmazásával pedig egy másik halfaj ragadozó ellenes adaptációjában kiemelkedő szerepű morfológiai tulajdonságok pontos genetikai hátterét sikerült feltárni (Peichel *et al*. 2001; Shapiro

et al. 2004). A példák és a genetikai/analitikai módszerek száma napról-napra növekszik. Aránylag ritka azonban a két módszer együttes használata egy adott modell-rendszeren, pedig a kettő jól kiegészíti egymást: amíg a *QTL mapping* alkalmazásával konkrét, számunkra valamiért érdekes tulajdonságok hátterét vizsgáljuk, a *genome scan* éppen a nem feltűnő, de az adaptációban fontos szerepet játszó génekre, és közvetve a génekhez rendelhető fenotípusos tulajdonságokra hívhatja fel a figyelmet. Ritka továbbá a *QTL mapping* alkalmazása olyan rendszerekben ahol nagyszámú ökológiailag releváns tulajdonság szétválása ismert, és éppen ezért nagyszámú tulajdonság térképezése lehetséges párhuzamosan. Egy ilyen megközelítés nem csak a hatékonysága miatt lehet jelentős, de a különböző tulajdonságok genetikai korrelációit (legyen az ok pleiotrópia vagy fizikai kapcsoltság) is közvetlenül a genom szintjén engedi tesztelni.

2. Célkitűzések

A disszertációmban bemutatott vizsgálatok célja a fajon belüli adaptív változatosság vizsgálata volt a tulajdonságok széles spektrumán. A problémához próbáltam minél több – hierarchikusan egymásra épülő – szinten nyúlni. Egy kistestű, rendkívül széles és változatos elterjedésű pikófajt, a kilenctüskés pikót választottam modellfajul (ennek okait lásd a faj ismertetésénél). Itt jegyezném meg, hogy gyakorlatilag az összes vizsgálatot kollegákkal és az általam vezetett diákokkal közösen végeztem. Erre a munka nagysága és az alkalmazott módszerek sokszínűsége miatt volt szükség. Bár szinte az összes konkrét vizsgálatban szervező-tervező szerepem volt, ott ahol a munka dandárját diák(ok), vagy az alkalmazott módszer specialistája végezte, természetesen ők a publikációk első szerzői.

A disszertáció (és az idetartozó vizsgálatok) három szintre oszthatóak. Első körben fel kívántuk tárni a különböző élőhelyeken előforduló populációk közötti habitat-függő fenotípusos szétválást, hogy elképzelésünk legyen a lokális adaptációkban érintett tulajdonságokról. A vizsgált tulajdonságok a morfológiától (testalak, tüskék, pajzsok) az életmeneten (testméret, növekedés, ivari dimorfizmus, fekunditás) és neurobiológiai tulajdonságokon (agyméret, oldalvonalszerv) át egészen a viselkedésig (aktivitás, kockázatvállalás, agresszió) terjedtek. Ezek a tulajdonságok jelentik a vizsgálatsorozat gerincét, ezekre épülnek a következő szintek. Ezeken kívül néhány, csak ebben a részben vizsgált tulajdonságot is összevetettünk populációk között, mint például a színlátás. Ahol az adatok engedték, az ivarok közötti különbségeket is vizsgáltuk.

A második részben a populációk élőhelyei közötti eltérés fő hatótényezőiként azonosított környezeti változók kísérletes manipulációjával indukált fenotipikus plaszticitást hasonlítottuk össze a populációk és az ivarok között. Itt a legfontosabb morfológiai, életmenet, neurobiológiai és viselkedési tulajdonságokon volt a hangsúly.

A harmadik rész fő célja az első részben habitat-függő populációs szétválást mutató tulajdonságok genetikai hátterének felderítése volt. Három megközelítést alkalmaztunk: először reciprok hibridek analízisén keresztül azonosítottuk a változatosságot létrehozó főbb komponenseket, majd egy speciális keresztezési protokollt (*inbred line cross design* [~ beltenyésztett vonal-keresztezés dizájn], Lynch & Walsh 1998) követve kísérletet tettünk a változatosságot befolyásoló genomszakaszok azonosítására *QTL mapping* módszerrel. Kiegészítésképpen a vizsgált tulajdonságoktól függetlenül is elvégeztünk egy *genome scan* vizsgálatot is abban a reményben, hogy beazonosítjuk azokat a genom szakaszokat ahol a természetes szelekció hatásai mérhetőek.

Mivel a populációs összehasonlítás alapját a közösségszerkezetbeli eltérések (egyes populációkban a kilenctüskés pikó az egyetlen halfaj, más populációban pedig egy gazdag, sok ragadozó halfajt is felvonultató közösség tagja) és az ebből adódó extrém predációs nyomásbeli különbség adta (részleteket lásd a vizsgált élőhelyek ismertetésénél), a populációs szétválást illető várakozásaink a következőek voltak:

- Az alacsony predációs nyomás alatt álló populációkban az intraspecifikus kompetícióban elért siker a rátermettség növelésének fő eszköze, ezért ilyen populációkban egy 'kompetitív fenotípus' ismételt, egymástól független megjelenését vártuk. Az általunk hipotetizált fenotípust redukált védekezési struktúrák, nagy felnőttkori testméret, gyors növekedés (térfogat/idő tekintetében) hosszan tartó növekedési időszakkal és akár késleltetett ivaréréssel, nagyobb fekunditás, kisebb agy, redukált oldalvonalrendszer és gyengébb színlátás, valamint magas agresszió és kockázatvállalás jellemezték.

A fenotipikus plaszticitás szerepe a lokális adaptációban egyelőre nem tisztázott (lásd 1.2). Függetlenül attól, hogy kvantitatív tulajdonságként vagy a lokális adaptáció felé vezető lépésként interpretáljuk, a releváns környezeti tényezők és azok populáción belüli változatossága alapján az alábbi hipotéziseket fogalmazhattuk meg:

- Az adott populáció-típus tagjai számára releváns környezeti hatás (pl. a ragadozó halakkal együtt élő pikók számára a becsült ragadozó-veszély) manipulációjával nagyobb plasztikus választ lehet indukálni ezekben a populációkban, mint azokban ahol a manipulált környezeti tényező kisebb jelentőségű.
- Amennyiben egy környezeti hatás az adott populáció-típusban térben és időben stabilan irreleváns (pl. a ragadozó halakat nem tartalmazó izolált tavakban élő pikók számára a ragadozó halak okozta veszély), az adott környezeti hatásra nem fogunk találni plasztikus választ.

- A válaszok erőssége az adott tulajdonság átlagos várt plaszticitásának megfelelően fog alakulni (általános morfológia < neurobiológiai képletek ≈ életmenet viselkedés).

A kvantitatív genetikai eredmények tekintetében konkrét hipotéziseket nem tudtunk megfogalmazni. Általánosságban a habitat-függő populációs szétválást mutató tulajdonságoknál feltételeztük, hogy kimutatható lesz valamilyen genetikai komponens. A *QTL mapping* vizsgálatoktól a legtöbb tulajdonság esetében a 'sok gén kis hatással' típusú mintázatot vártuk, a *genome scan*-nél pedig a lehető legtöbb szelekció alatt álló régió azonosítását reméltük.

3. Anyag és módszer

A disszertációmban tárgyalt kutatások módszertana igen széles, a viselkedési megfigyelésektől a retina fényelnyelésének mérésén és az alak számszerűsítésére használt geometriai morfometrián át egészen a kurrens genetikai és genomikai módszerekig terjed. Ezért a módszertan kimerítő ismertetésétől eltekintek, és azt csak a dolgozat megértéséhez szükséges mélységben teszem meg (ez különösen igaz a genetikai és statisztikai módszerekre). Ugyanakkor minden módszertani részlet megtalálható a disszertáció végén lévő Függelékben (9), ahol a disszertációban említett vizsgálatokat feldolgozó folyóiratcikkeket és kéziratokat csatoltam. A disszertációban csak olyan eredmény szerepel, ami a Függelékben bemutatott publikációkban is megtalálható.

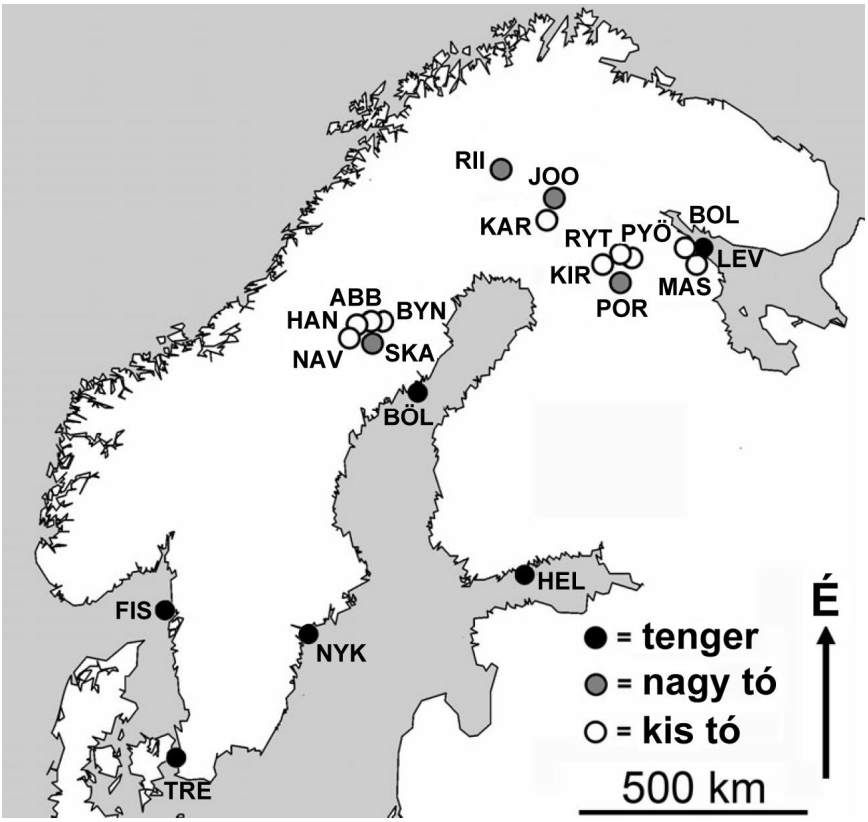
3.1 A vizsgált faj

A kilenctüskés pikó (*Pungitius pungitius* Linnaeus 1758) a Gasterosteiformes rend Gasterosteidae családjába tartozó kistestű csontoshal (1. ábra). Rendkívül széles elterjedési területtel bír az északi féltekén, és meglepően változatos élőhelyeken találkozhatunk vele a tengerek partvidékétől kezdve a nagy folyó- és tórendszereken át az egészen kis patakokig, csatornákig (Bănărescu & Paepke 2001; Östlund-Nilsson et al. 2007). A fajra jellemző, hogy képes hosszú távon stabilan megmaradni izolálódott tavakban (melyek felülete akár az 5 hektárnál is kisebb lehet; 2. ábra) egyetlen halfajként (Bănărescu & Paepke 2001). Általában 5-6 cm hosszúra nő. A hátán 7-11 kicsiny tüskét, a mellúszói alatt-mögött pedig egy pár jól fejlett tüskét visel, melyeket fixen „ki tud pattintani" a tüskék körüli csontos struktúráknak köszönhetően. A testének feji és farki végén pajzsokat figyelhetünk meg. Noha a kilenctüskés pikónál megfigyelt morfológiai védelmi struktúra gyengébb a háromtüskés pikóénál (*Gasterosteus aculeatus*), még mindig egyértelmű előnyt jelent a védelmi struktúrákat nem viselő halakhoz képest (Hoogland et al. 1957).

1. ábra. Kifejlett nőstény kilenctüskés pikók (*Pungitius pungitius*). Az alsó példány Helsinki közeléből, a Balti-tengerből, a felső pedig a Rytilampi nevű észak-finn izolált kis tóból származik. A pontos előfordulási helyeket a 3. ábrán és az 1. táblázatban adom meg.

2. ábra. Tipikus izolált kis tó. A fényképen Bynastjärnen (lásd 3. ábra, 1. táblázat) látható.

Az általunk vizsgált pikófaj közeli rokona a háromtüskés pikónak, amely az evolúcióbiológia kedvelt modellfaja, amit a fajra koncentráló számos könyv is bizonyít (pl.: Wootton 1976, 1984; Bell & Foster 1994; Östlund-Nilsson et al. 2007). Noha a kilenctüskés pikó általános ökológiájáról és a háromtüskés pikóéval vetekedő morfológiai változatosságáról a rendelkezésünkre állnak adatok (Jones & Hynes 1950; McPhail 1963; Gross 1979; Blouw & Boyd 1992; Ziuganov & Zotin 1995; Heins et al. 2003, 2005) a róla szóló evolúcióbiológiai ismeretek meglehetősen hiányosak. Azonban néhány, a háromtüskés pikóval való összevetésen alapuló funkcionális genomikai vizsgálat (Shapiro et al. 2006, 2009) a kilenctüskés pikó kutatásában rejlő potenciált sejteti. A faj már az általa benépesített élőhelyek sokfélesége és a morfológiai változatossága (McPhail 1963; Gross 1979) alapján is méltó a tudományos figyelemre, a jelentőségét tovább növeli a potenciális szerepe a konvergens evolúció megértésében. A kilenc- és háromtüskés pikó több mint 10 millió éve vált szét (Shapiro et al. 2006; Bell et al. 2009; Aldenhoven et al. 2010). A jelenlegi elterjedési területeik és élőhelyeik azonban jelentősen átfednek, és a posztpleisztocén történetük (az utóbbi 10-15000 év) is hasonló. Mivel a háromtüskés pikóra kidolgozott módszertan (a fogságban szaporítástól a genomikai megközelítésekig) könnyen adaptálható a kilenctüskés pikóra is, ez a fajpár könnyen válhat a konvergens evolúció vizsgálatának kiemelt modelljévé (Merilä 2013).



3. ábra. A vizsgálataimban felhasznált populációk. A rövidítéseket az 1. táblázatban adom meg. Értelemszerűen nem minden vizsgálatban szerepelt minden felsorolt populáció, a részletek a Függelékben csatolt, az egyes vizsgálatokat részletesen taglaló folyóiratcikkekben és kéziratokban találhatóak. A különböző *common garden* kísérlettekben a FIS, NYK, BÖL, HEL, LEV, ABB, BYN, PYÖ és RYT populációk szerepeltek, ismét csak változó kombinációkban.

3.2 A vizsgálati terület

A vizsgált populációk a fennoskandináviai területen vannak (3. ábra), Svédországban, Finnországban és Oroszországban, meglehetősen nagy területet lefedve. Három habitattípust reprezentálnak: tengerek partvidéke, nagy tavak és apró, izolált tavacskák. A hangsúly főleg a tenger-partvidék és az apró tavak (2. ábra) összehasonlításán volt, mivel ezek a habitattípusok jelentik a kilenctüskés pikó élőhelyeinek extrém változatait. A fő különbségként a halközösségek szerkezetét azonosítottuk. A tengerekben (és a nem izolált édesvízi élőhelyeken) előforduló kilenctüskés pikók egy nagy diverzitású halközösség tagjai. Itt sok és változatos ragadozó halfajjal (lazacfélék [Salmonidae], csuka [*Esox lucius*], csapósügér [*Perca fluviatilis*] és süllő [*Sander luciperca*] – megjegyzendő, hogy a parthoz közeli alacsonyabb sótartalmú helyeken az alapvetően édesvízi fajok is nagy számban előfordulnak) élnek együtt, csakúgy, mint rengeteg potenciális versenytárssal, mint például a háromtüskés pikóval. Ezzel szemben a tipikus izolált kis tóban a kilenctüskés pikó az egyetlen halfaj.

Ezekből a különbségekből kiindulva feltételeztük, hogy a két habitattípus között a fő szelekciós különbséget a szimpatrikus ragadozóhalak jelenléte-hiánya, illetve a ragadozóhalak és interspecifikus kompetitorok hiánya esetén előtérbe kerülő intraspecifikus verseny erőssége jelentik. Természetesen ennyire eltérő élőhelyek esetén a potenciálisan különböző releváns környezeti faktorok száma magas, többek között a sótartalom jut azonnal az ember eszébe. A mi feltételezésünket támogatták azonban az elővizsgálataink múzeumi és a kutatócsoport (Ecological Genetics Research Unit, Helsinki Egyetem, Finnország) saját gyűjteményében lévő preparált egyedeken, illetve a fogságban tartás és szaporítás több populáción való kipróbálásánál élő példányokon. Külsőleg a tengeri, nagy tavi, folyóból vagy kis patakból származó pikók mind egyformának tűntek, különösen az izolált kis tavakból való egyedekkel összehasonlítva. Ez utóbbiak gyakorlatilag más fajnak látszanak az avatatlan szem számára (1. ábra). Az előzetes viselkedési megfigyelések is hasonló képet festettek: a tengeri, nagy tavi vagy kis patakból gyűjtött halak a szaporodási fázisban lévő hímek kivételével semmi, vagy csak minimális agressziót mutattak egymással szemben, míg az izolált kis tavakból származó pikóknál akár a nőstények is igen rövid idő alatt végeztek egymással. Megjegyzendő továbbá, hogy ahol később a nagy tavakból származó minta is analízisre került, mindig a tengeri mintákhoz való hasonlóságukat találtuk, azaz az izolált kis tavakból származó egyedek egyértelműen elkülönültek a többi habitattípustól (Herczeg et al. 2009a; 2010a). Végezetül, azoknál az izolált kis tavaknál ahol a feltételezett környezeti különbség nem állt fent, mert – akár kompetitor, akár ragadozó – más halfaj is megtalálható volt a kilenctüskés pikó mellett, a megfigyelt fenotípus közelebb állt a tengeri, mint a tipikus izolált kis tavi fenotípushoz (Herczeg et al. 2009a; 2010a). Ez az érvelés természetesen nem bizonyító erejű, de mivel a természet nem hozott létre faktoriális elrendezést, azaz nem voltak izolált kis tavak diverz halfaunával, és főleg nem voltak tengeri vagy nagy tavi élőhelyek kizárólag kilenctüskés pikóval, az elméletünkre, miszerint a

szelekciós erők fő különbsége a közösségszerkezet eltérésén alapul csak közvetett bizonyítékaink vannak (a 4.1 részben felsorolt eredmények legnagyobb része).

A különböző vizsgálatok gyakran eltérő (de nagyrészt erősen átfedő) populációkra támaszkodtak. A bemutatott térképen (3. ábra) az összes felhasznált populációt feltüntettem a habitattípussal együtt, valamint kiemeltem a *common garden* kísérletekbe vont populációkat is. A vizsgált populációk releváns tulajdonságait táblázatos formában is bemutatom (1. táblázat). Az adott vizsgálatban részt vevő populációk listája (amennyiben a disszertációban nincs megadva) mindig megtalálható a disszertáció végén lévő Függelékben (9) csatolt folyóiratcikkekben vagy kéziratokban.

3.3 A halak gyűjtése, konzerválása

A szaporításhoz vagy vizsgálatokhoz szükséges kifejlett példányokat javarészt a szaporodási időszak előtt-elején gyűjtöttük, hogy lehetőleg a nőstények első ikrázásából tudjunk szaporítani, és a morfológiai vizsgálatainkat ne befolyásolja a nőstények ikrával való teltsége. Kétféle módszert alkalmaztunk. Ahol a terepviszonyok lehetővé tették, ott kisméretű, kézi kerítőhálót (*seine net* vagy *beach seine*) használtunk, illetve iszapos, akadós fenékviszonyoknál, vagy amikor sok víztestben párhuzamosan próbáltunk gyűjteni, akkor kis fém varsákat (*minnow trap*) helyeztünk ki és ellenőriztünk minimum egyszer naponta. Amennyiben a befogott pikókra élve volt szükségünk, akkor azokat 20 literes levegőztetett és hűtött műanyag kannákban szállítottuk a Helsinki Egyetem halszaporító és –tartó laborjába. Ha viszont az állatokból preparátum készült morfológiai mérésekhez, akkor először túlaltattuk őket MS222 (*tricaine methanesulfonate*) altatóval, majd körülbelül két hónapig 96%-os etanolban tároltuk őket, ami után egy legalább két hetes fixáció következett 4%-os formalinban. A fixáció előtt DNS mintákat vettünk, gyakorlatilag a két mellúszót vágtuk le és helyeztük 96%-os etanolba, majd tároltuk –20°C-on. Ez után került sor a csontok színezésére, amire különösen a pajzsok számolásánál volt szükség. Ehhez Pritchard & Schluter (2001) *Alizarin Red S* protokollját követtük.

3.4 Fogságban tartás, szaporítás és kezelések

Az esetek túlnyomó részében a szaporítást laborban, *in vitro* végeztük. Ehhez a természetből gyűjtött ivarérett halakat értelemszerűen először szaporodási kondícióba kellett hozni. A halakat *ad libitum* etettük fagyasztott vörös szúnyoglárvával (Chironomidae sp.) és 15-17°C-on tartottuk folyamatos megvilágítást alkalmazva, hogy a magas szélességi övek természetes nyári viszonyait imitáljuk. A hímek szaporodási kondícióját a hasi tüskéik opálos-fehéres színe jelezte (a hasi fekete nászszínezetet csak a domináns hímek mutatták), a nőstényeknél a has teltsége mellett a has enyhe nyomására a kloáka-nyílásban megjelenő szabályos, kerek ikrák megléte volta biztos jel. Amennyiben volt megfelelő tenyészpár, a hímeket túlaltattuk MS222-vel, a heréiket kiemeltük és egy csepp vízben elmorzsoltuk. Az ikrás nőstényektől az ikracsomót a hasi régióra kifejtett enyhe

Mintavételi terület	Rövidítés	Koordináták	Terület (ha)	Szimpatrikus halfajok
Tenger				
Fiskebäckskill, Atlanti-óceán, Svédország	FIS	É 58°24’; K 11°47’	---	Komplex közösség
Trelleborg, Balti-tenger, Svédország	TRE	É 55°38’; K 13°12’	---	Komplex közösség
Nyköping, Balti-tenger, Svédország	NYK	É 58°43’; K 17°03’	---	Komplex közösség
Bölesviken, Balti-tenger, Svédország	BÖL	É 63°39’; K 20°12’	---	Komplex közösség
Helsinki, Balti-tenger, Finnország	HEL	É 60°13’; K 25°11’	---	Komplex közösség
Levin Navolok, Fehér-tenger, Oroszország	LEV	É 66°18’; K 33°25’	---	Komplex közösség
Nagy tó				
Porontima, Finnország	POR	É 66°12’; K 29°16’	115	Komplex közösség
Joortilojärvi, Finnország	JOO	É 66°49’; K 26°34’	20	Pénzes pér, *Thymallus thymallus*
Riikojärvi, Finnország	RII	É 68°06’; K 23°34’	20	Csuka, *Esox lucius*, Nagy maréna, *Coregonus lavaretus*
Västre-Skävtrasket, Svédország	SKA	É 64°26’; K 19°27’	35	Komplex közösség
Kis tó (izolált)				
Bolotnoje, Oroszország	BOL	É 66°18’; K 33°25’	< 5	Háromtüskés pikó, *Gasterosteus aculeatus*
Mashinnoje, Oroszország	MAS	É 66°18’; K 33°25’	< 5	*G. aculeatus*
Pyöreälampi, Finnország	PYÖ	É 66°15’; K 29°26’	< 5	*C. lavaretus*
Rytilampi, Finnország	RYT	É 66°23’; K 29°19’	< 5	-
Kirkasvetinenlampi, Finnország	KIR	É 66°26’; K 29°08’	< 5	Sebes pisztráng, *Salmo trutta*
Karhulampi, Finnország	KAR	É 66°39’; K 26°26’	5,8	*S. trutta*, *C. lavaretus*
Abbortjärn, Svédország	ABB	É 64°29’; K 19°26’	< 5	-
Bynästjärnen, Svédország	BYN	É 64°27’; K 19°26’	< 5	-
Hansmyrtjärn, Svédország	HAN	É 64°33’; K 19°10’	< 5	*S. trutta*
Lil-Navartjärn, Svédország	NAV	É 64°33’; K 19°11’	< 5	?

1. táblázat. A vizsgálataimban felhasznált populációk. Értelemszerűen nem minden vizsgálatban szerepelt minden felsorolt populáció, a részletek a Függelékben (9) csatolt, az egyes vizsgálatokat részletesen taglaló folyóiratcikkekben és kéziratokban találhatóak. A különböző *common garden* kísérlettekben a FIS, NYK, BÖL, HEL, LEV, ABB, BYN, PYÖ és RYT populációk szerepeltek, ismét csak változó kombinációkban.

nyomással nyertük ki (ezt a nőstények minden esetben károsodás nélkül átvészelték). Ezek után a spermaoldatot a petecsomóra cseppentettük. Körülbelül 30 perc várakozási idő után az ikracsomót vízbe helyeztük és a kikelésig (5-6 nap) naponta ellenőriztük sztereomikroszkóp alatt, hogy a terméketlen ikrát eltávolítsuk. A kikelés után 4-5 nappal a petezsák felszívódik, a halak önállóan úsznak és megkezdik a táplálkozást.

A kísérletekben alkalmazott kezelések ebben az életkorban kezdődtek. Amikor nem volt kezelés (illetve a kezeléses vizsgálatok kontrolljainál), a kiválasztott halakat egyedileg elhelyeztük az *Allentown Zebrafish Rack System* (Aquaneering Inc. San Diego, CA) nevű zárt rendszer 1,4 literes tartályaiba. A rendszerben keringetett vizet fizikai, kémiai, biológiai és UV szűrés tartotta tisztán. A tartályok közötti átláthatóságot megszűntettük, így a halak a fajtársaktól és ragadozóktól származó ingerek nélkül fejlődhettek. Táplálékul kezdetben élő sórák (*Artemia salina*) nauplius-okat, majd fagyaszott evezőlábú rákokat (*Cyclops* sp.) és végül fagyasztott vörös szúnyoglárvat kaptak. A hőmérséklet és fényviszonyok kísérletek között eltérhettek (lásd Függelék, 9). A kísérletekben háromféle kezelést alkalmaztunk. A táplálék-kezelés egyszerű volt, az alacsony táplálék kezelésben a pikók minden második nap egyszer, míg a magas táplálék kezelésben naponta kétszer kaptak táplálékot *ad libitum*. A ragadozó-kezelés a ragadozó szaganyagának jelenlétén-hiányán alapult. Ragadozóként csapósügereket használtunk. Ezek az alapvetően édesvízi halak a kilenctüskés pikó közönséges ragadozói, és nemcsak a Fennoskandináv édesvizek talán legközönségesebb halai, de az alacsony sótartalmú Balti-tengerben és általában az északi parti régiókban is igen gyakoriak (Koli et al. 1988; Ådjers et al. 2006). A kezelésekhez minden rendszerhez csatlakoztattunk egy 150 literes külső tartályt, amibe vagy raktunk két 10-15 cm hosszú csapósügeret vagy csak tiszta vizet töltöttünk. A szociális kezelés esetében a halak nem a fenti rendszerben fejlődtek. Itt kezdetben 10 literes tartályokba került körülbelül 20-40 hal (az adott család egyedszámától függően), majd a családok populáción belüli egyenletes keverése után 100 halas csoportokat neveltünk 140 literes tartályokban.

3.5 A főbb vizsgált változók

3.5.1 Morfológia

Morfológiai szempontból két komplex tulajdonságot vizsgáltunk: a testalakot és a ragadozó elleni csontos védelmi struktúrákat. A testalak vizsgálata hosszú múltra tekinthet vissza és az elmúlt évtizedben az *a priori* kiválasztott lineáris változók (pl. különböző képletek hossza, szélessége) helyett egyre inkább teret hódított az iránypont-alapú geometriai morfometria (*landmark-based geometric morphometrics*), ahol a testalakot egységes tulajdonságként kezeljük minimális információvesztés mellett (Bookstein 1991; Zelditch et al. 2004). Az adatgyűjtés alapját a halak oldaláról készített digitális fényképek és a halak körvonalában található fix pontokra (pl. csontos képletek vagy uszonyok kezdetének-végének helyei) digitálisan helyezett iránypontok jelentették. Az adatok kinyeréséhez és

analíziséhez a tpsDig és tpsRelw szoftverek aktuális verzióit használtuk (Rohlf 2006; a szoftverek ingyenesen letölthetőek a következő címről: http://life.bio.sunysb.edu/morph/).

A csontos védelmi struktúrák közé az alábbi képletek tartoznak: a hasi tüske (*pelvic spine*), a mell-öv (*pelvic girdle*) és a pajzsok (*lateral plate*). A pajzsok mérete a kilenctüskés pikónál kicsiny és a háromtüskés pikóval ellentétben már csak egészen minimális szerepe lehet a védelemben. Ennek ellenére történeti és evolúciós okokból a hasi tüskével és mell-övvel együtt tárgyalom a pajzsokat is. A hasi tüskéket digitális tolómérővel mértük 0,01 mm pontossággal. Három mérés átlagát használtuk a mérési hiba csökkentése érdekében. A mell-öv hosszát a digitális fényképeken mértük a tpsDig szoftverrel (Rohlf 2006). A pajzsok számát sztereomikrószkóp használatával rögzítettük.

3.5.2 Életmenet

A standard testhosszt (orrcsúcstól a faroknyél végéig) használtuk a hossz mérésére. A mérésekhez digitális fényképeket és az aktuális tpsDig szofvert (Rohlf 2006) használtuk. A testtömeget digitális mérlegen 0,01 g pontossággal mértük vízben. A klasszikus testhosszon és testtömegen kívül még egy változót használtunk a testméret leírására, a centroid méretet (*centroid size*). Kiszámítása az iránypontok által meghatározott felület centroidja és az iránypontok távolságainak négyzetösszege a gyök alatt (Bookstein 1991). Ez a változó általában erősen korrelál a standard testhosszal, de figyelembe veszi az állat „vaskosságát” is, azaz a testhosszal szemben nem egy dimenziós.

A különböző életkorban mért testméretekből növekedési rátát is kalkuláltunk. Mivel azonos korú halaknál az egyszerű tömeg/idő jellegű ráták semmi extra információt nem hordoznak az azonos életkorban mért nyers adatokhoz képest, a von Bertalanffy növekedési görbe (von Bertelanffy 1938) paramétereit használtuk a növekedési stratégia leírásához. A von Bertalanffy növekedési görbe három paramétert ad meg, a kiindulási méretet, az aszimptotikus maximális méretet és a növekedési konstanst. A mi esetünkben, ahol a kiindulási méret ismert (kísérletes adatok) csak a másik két paraméterek van biológiai relevanciája. Fontos kiemelni, hogy a növekedési konstans eltér a klasszikus növekedési rátától, azt írja le, hogy milyen gyorsan telítődik a görbe, azaz milyen gyorsan éri el a közel maximális méretét az egyed.

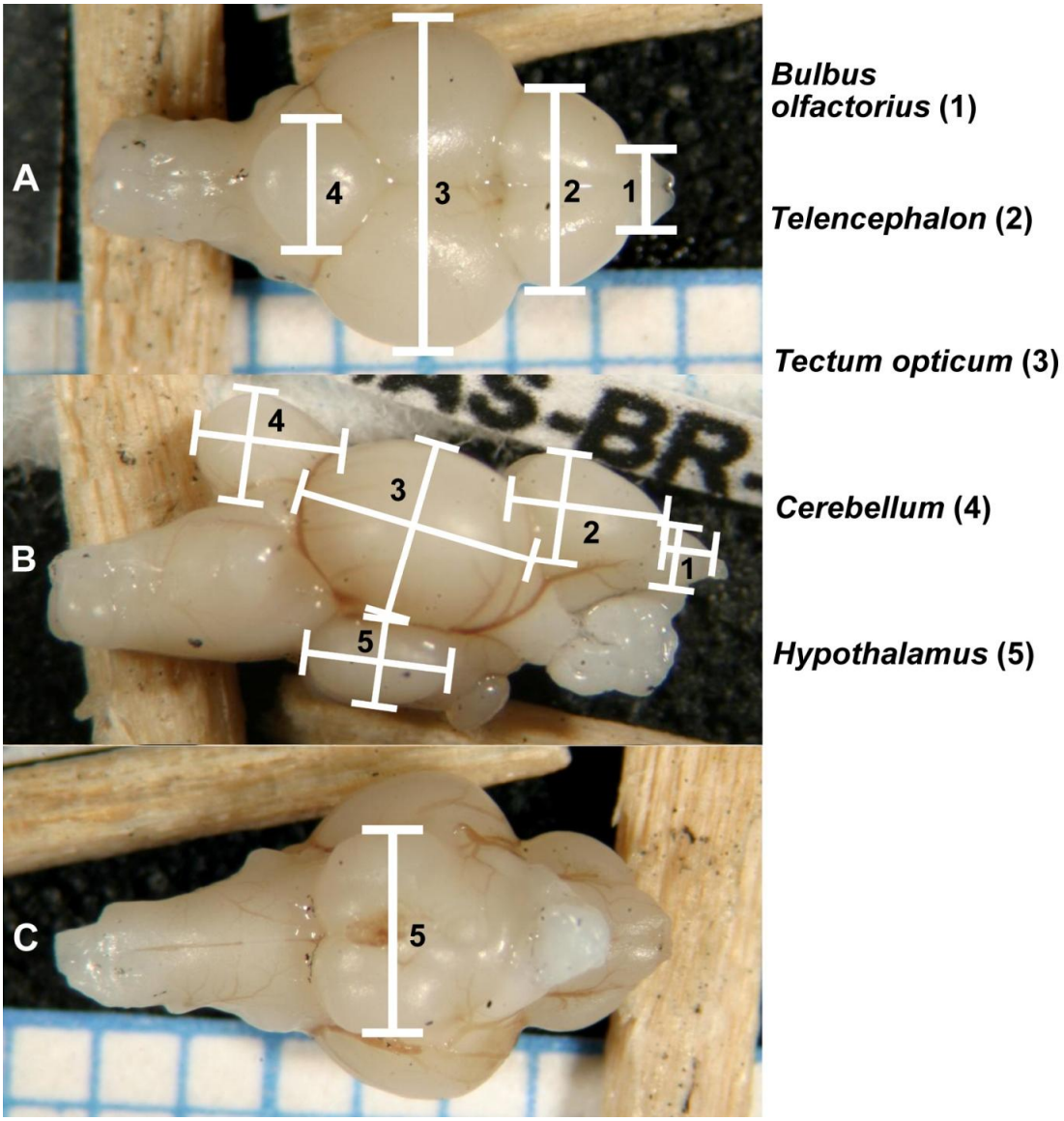
A halak korának meghatározását az úszósugarakban megtalálható, a fák évgyűrűihez hasonlító gyűrűk alapján végeztük. Az úszósugarakat a tövükhöz közel elvágtuk, neutrális vörös festéssel színeztük és a gyűrűket mikrószkóp alatt számoltuk. Az így kapott becslésünk megbízhatóságát az otolitokban talált gyűrűk számához (Shirvell 1981) hasonlítva teszteltük egy almintán: a két módszerrel becsült életkor megegyezett.

Az ivarérés korának becsléséhez mestersége hibernációt vezettünk be (4°C és teljes sötétség) a *common garden* kísérletben. Az így kapott becslések természetesen csak összehasonlításra voltak jók, a természetes ivarérettségi életkort nem reprezentálták pontosan. A fekunditást az ikrák számával és méretével jellemeztük. Az ikraméretet az ikrákról készített digitális fényképekről mértük (a kör átfogója) a tpsDig szoftverrel (Rohlf 2006).

Az energiaraktárak becslésére három változót használtunk. Először mértük a belső szerv-mentes testtömeget (*eviscerated body mass*). Ez a változó természetesen nem becsli az energiaraktárakat tisztán. A relatív izom- és csonttömeg azonban általánosan jellemzi a kondíciót és jó indikátora az izmokban tárolt glikogén és zsír mennyiségének is (Chellappa et al. 1989; Huntingford et al. 2001). A második változónk az úgynevezett zsírtest (*fatbody*) tömege volt. Ez a testüregben található, összefüggő képletet alkotó zsírtömeg. Utoljára mértük a máj tömegét. A máj fontos zsír- és glikogéntár és ezért a méretét az energiaraktárak indikátoraként használhatjuk (Chellappa et al. 1995). Mindhárom változót digitális mérleggel, 0,01 g pontossággal mértük.

3.5.3 Idegrendszer, érzékszervek

Az agyméret becslésének számtalan metódusa ismert a fej térfogatának mérésétől (Iwaniuk & Nelson 2002; Møller 2010) a teljes agyból készített metszetsor digitalizálásán alapuló térfogat becslésig (Airey & DeVoogd 2000; Wilson & McLaughlin 2010). Bár kétségtelenül ez utóbbi adja a legprecízebb becslést, a módszer anyag- és főleg időigénye nem teszi lehetővé nagyobb számú egyed gyors mérését. Van azonban egy gyorsabb módszer, az agy három oldalról való lefényképezésén és a fényképekről három dimenzióban mért lineáris méreteken alapuló térfogatbecslés. Az ellipszoid modellt (az agyat és annak részeit ellipszoid alakkal közelíti) alkalmazva Pollen et al. (2007) be tudta bizonyítani, hogy az így kapott térfogatok erősen korrelálnak a metszet-sorozatokon alapuló módszer térfogatbecsléseivel. Ezért mi is Pollen et al. (2007) módszerét követtük. Az MS222-val túlaltatott állatok agyát frissen kiboncoltuk és 4%-os pufferelt formalinban fixáltuk minimum 48 óráig. Ezek után az agyakról három nézetből (felülről, oldalról és alulról) digitális fényképeket készítettünk. A fényképekről az alábbi képletek hosszát, szélességét és magasságát mértük a tpsDig szoftverrel (Rohlf 2006): *bulbus olfactorius*, *telencephalon*, *tectum opticum*, *cerebellum*, *hypothalamus* (4. ábra) és a teljes agy. Ezekből az adatokból az ellipszoid modell alkalmazásával becsültük az adott képlet térfogatát (Huber et al. 2007; Pollen et al. 2007). Az így kapott becslések magas repetabilitást mutattak ($R > 0,8$; Gonda et al. 2009a,b).



4. ábra. A kilenctüskés pikó agya, dorzális (A), laterális (B) és ventrális (C) nézetből. Az ábrán látszanak a különböző vizsgált agyterületek, és az azok térfogatának becsléséhez szükséges három dimenziót lefedő mérések.

A mechanoreceptoros oldalvonalszerv a halak és vízi kétéltűek speciális vízmozgásokat érzékelő szerve (Dijkgraaf 1963). Az oldalvonalszerv funkcionális alapegysége a neuromaszt, ami lehet a testfelszínen, és lehet kis folyadék által kitöltött csatornákban a bőrben. Típustól függetlenül jól elkülönülő sorokba rendeződnek (oldalvonalak) főként a fej környékén, és aránylag könnyen számolhatóak sztereomikroszkóp alatt. A neuromasztok számolhatóságát elősegítik a különböző festési eljárások. Mi a már fixált mintákon *Alizarin Red* festést (Trokovic et al. 2011a, 2012), míg a még élő halakon *DASPEI* eljárást (Välimäki 2012, Välimäki, Herczeg, Trokovic, Merilä, kézirat) alkalmaztunk.

A színlátást, azaz pontosabban fogalmazva a retina látópigmentjeinek fényelnyelési spektrumát mikrospektrofotometriával mértük. Röviden, az elő halakat több órán át teljes sötétben tartottuk, majd dekapitáltuk őket. Az egyik szemet

eltávolítottuk, a retina központi részét leválasztottuk és roncsoltuk, hogy a fotoreceptorokat izolálni lehessen. A megfelelően preparált mintát a Helsinki Egyetemen épített egysugarú, számítógép-vezérelt, gyors hullámhossz-letapogató mikrospektrofotométerrel (*single-beam, computer-controlled, fast wavelength-scanning microspectrophotometer*; Govardovskii et al. 2000) analizáltuk.

3.5.4 Viselkedés

Kontextus és szituáció alapján elkülönítve négy viselkedést mértünk: táplálkozási aktivitást ismert környezetben zavarás nélkül (táplálkozási aktivitás); táplálkozási aktivitást ismert környezetben zavarást követően (kockázatvállalás táplálkozási kontextusban), agressziót fajtárs ellen (agresszió), és a búvóhely elhagyását új környezetben, zavarást követően (kockázatvállalás explorációs kontextusban). A viselkedési formák megbízható elkülönítése, azaz annak az eldöntése, hogy ugyanazt a viselkedést merjük más változóval, vagy funkcionálisan eltérő viselkedéseket mérünk notóriusan nehéz. Bár Réale et al. (2007) az emberi személyiségvizsgálatok analógiájára elkülönített öt fő viselkedési tengelyt, mára ennek az *a priori* elkülönítésnek a megbízhatósága megkérdőjeleződött (Garamszegi et al. 2013). Ezért a négy változónk közötti kapcsolat mibenlétének intuitív magyarázatára nem tennék kísérletet, a változókat az adott vizsgálat kérdéseinek megfelelően külön és együtt, viselkedési típusként is analizáltuk.

3.6 Genetikai vizsgálatok

A populációs szétválás mögötti potenciális komponensek szerepét egy kvantitatív genetikai kísérlettel kívántuk tisztázni. A módszer régóta ismert (Wright 1978) és – ha nem is gyakran – alkalmazott (Laugen et al. 2002). A lényege, hogy két, a természetben különböző fenotípusokat tartalmazó populációból létrehozunk „tiszta” és hibrid vonalakat *common garden* kísérletben, úgy, hogy a két populációból származó hím és nőstény egyedeket minden variációban párosítjuk. A predikciók viszonylag egyszerűek. Amennyiben a két tiszta vonal különbözik, a direkt környezeti indukció (fenotipikus plaszticitás) mint egyetlen magyarázat elvethető. Amennyiben a hibridek a két tiszta vonal között nagyjából félúton helyezkednek el és egymástól nem különböznek, az additív genetikai komponens a fő. Amikor a hibridek az egyik tiszta vonalhoz hasonlítanak, domináns genetikai hatásról beszélhetünk. Ha a hibridek az anyjuk populációjához hasonlítanak, az anyai hatás a döntő. Amennyiben a laborvonalak egyformák, és a természetes populációk közötti fenotípust mutatnak, a genetikai hatásoktól mentes direkt környezeti indukció (fenotipikus plaszticitás) a természetben megfigyelt változatosság oka. Ennél jobb felbontású eredményekhez még egy generációt kell létrehozni az összes létező hibrid és tiszta vonalakkal (Huttunen & Aspi 2003), ami már csak kevés fajnál reális és a kis izolált tavakból származó kilenctüskés pikók késleltetett ivarérése (Ab Ghani et al. 2013) miatt nekünk nem sikerült. A módszer részletesebb leírását a 4.3.1 pontban adom meg.

A DNS alapú genetikai módszereink túlnyomórészt standard PCR-alapú mikroszatellita analízisek voltak (Shikano et al. 2010c, 2013; Trokovic et al. 2011a, 2012; Laine et al. 2012a, 2013, 2014; Karhunen et al. 2014). Mitochondriális gén és nukleáris gén szekvenálására csak egyedi esetekben került sor (Shikano et al. 2010c; Saarinen et al. 2012). A *genome scan* vizsgálatunkban egy új genom komplexitást redukáló módszert fejlesztettünk ki, amit *paired-end double restriction-site-associated DNA* protokollnak neveztünk el (Bruneaux et al. 2013).

3.7 Statisztikai analízisek

A adatelemzések többségét a lineáris modellek családjába tartozó próbastatisztikák jelentették: általános lineáris modellek (*General Linear Model*; GLM), ezek nem-normál eloszlású adatokra alkalmazott változatai, az általánosított lineáris modellek (*Generalized Linear Model*; GLM), illetve a random hatásokat is figyelembe vevő általános lineáris kevert modellek (*General Linear Mixed Model*; GLMM) és ezek nem-normál eloszlású adatokra alkalmazott változatai, az általánosított lineáris kevert modellek (*Generalized Linear Mixed Model*; GLMM). Ezeknek használtuk a többváltozós (*multivariate*) és ismételt méréses (*repeated measures*) változatait is. Amikor a válaszváltozóink nem voltak függetlenek, dimenzióredukciót is alkalmaztunk. Ehhez főkomponensanalízist (*Principal Component Analysis*; PCA) használtunk. Különböző korrelációkat, t-próbákat, es egyszerűbb nemparametrikus próbákat is gyakran alkalmaztunk. Ezeket a statisztikákat a SAS (SAS Institute Inc., Cary, USA), az SPSS (SPSS Inc., Chicago, USA) és a STATISTICA (StatSoft Inc.,Tulsa, USA) szoftvercsomagok aktuális verzióival végeztük. Egyes speciális statisztikai problémák kezelésére felhasználtuk még többek között az R (R Development Core Team 2007), az MCMCglmm (Hadfield 2010), a MrBayes (Ronquist & Huelsenbeck 2003), az FSTAT (Goudet 2001), az Arlequin (Excoffier et al. 2005), a CRI-MAP (Green et al. 1990) és a GridQTL (Seaton et al. 2006) programcsomagok aktuális verzióit is.

4. Eredmények és megvitatásuk

Az egyes vizsgálatokhoz kötődő eredmények részletes bemutatását és részletekbe menő megvitatását a Függelékben (9) csatolt folyóiratcikkek és kéziratok tartalmazzák. A disszertációmban egyrészt a főbb egymásra épülő eredményeket, másrészt az érdekességeket tárgyalom, gyakran egy adott vizsgálat eredményei közül csak bizonyos részeket ragadva ki, esetenként az adott publikációétól eltérő szempontból értékelve. A dolgozat 28 publikált folyóiratcikk és 3 kézirat (azaz 31 függetlenül is értelmezhető kutatás, 7, 9) eredményeit mutatja be. Az összes vizsgálat statisztikai analízisének pontos módszertani leírása terjedelmi okokból nem került bele a dolgozatba. Ezek nélkül viszont egy-egy kiragadott statisztikai próba eredményeinek a szövegbe szúrása értelmezhetetlen lenne, és csak az olvashatóságot rontaná. Ezért az eredményeket verbálisan ismertetem és állításokat csak a

statisztikai eredményeket figyelembe véve fogalmazok meg. A statisztikai módszertan és a statisztikai próbák eredményei teljes egészében megtalálhatóak a Függelékben csatolt folyóiratcikkekben és kéziratokban.

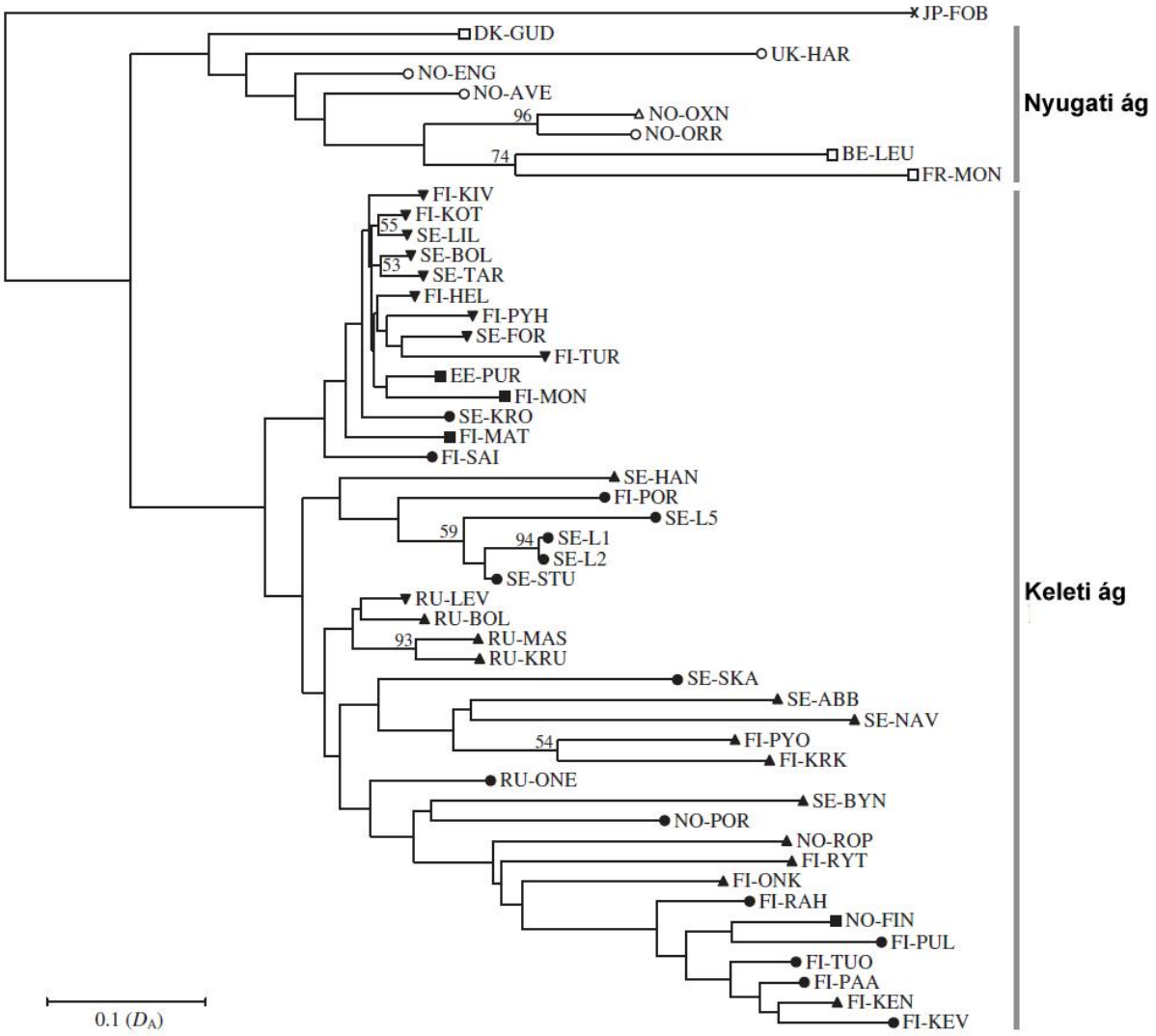
4.1 Populációs különbségek

Ebben a fejezetben a populációk közötti habitat-függő változatosságot veszem górcső alá, nagyszámú populációból gyűjtött minta alapján. Ahol lehetett, ott kiegészítem az eredményeket kevesebb populáció *common garden* kísérletből származó adatai alapján, hogy közelebb kerüljünk a genetikai háttérrel rendelkező mintázatokhoz és kitértem az ivari dimorfizmus vizsgálatára is.

4.1.1 Populációgenetikai elővizsgálat

A disszertációmban összefoglalt vizsgálatok egyik közös vonása, hogy egy faj populációinak összehasonlításán alapszanak. Az ilyen kutatásoknál kritikus fontosságú feltérképezni a vizsgálni tervezett populációk genetikai struktúráját, elkerülendő a különböző genetikai leszármazási vonalakba, vagy esetleg akár (al)fajokba tartozó populációk egyenrangú egységekként való kezelését. A kilenctüskés pikó az északi féltekén cirkumpoláris elterjedést mutat, és szinte minden vízi élőhely-típusban megtalálható (Wooton 1976; Bănărescu & Paepke 2001). Mivel Észak-Európát körülbelül 12000 évvel napjaink előtt még a kontinentális jégtakaró borította, a jelenlegi északi populációk a jégkorszakot déli refúgiumokban átvészelő ősök posztglaciális rekolonizációjának eredményei.

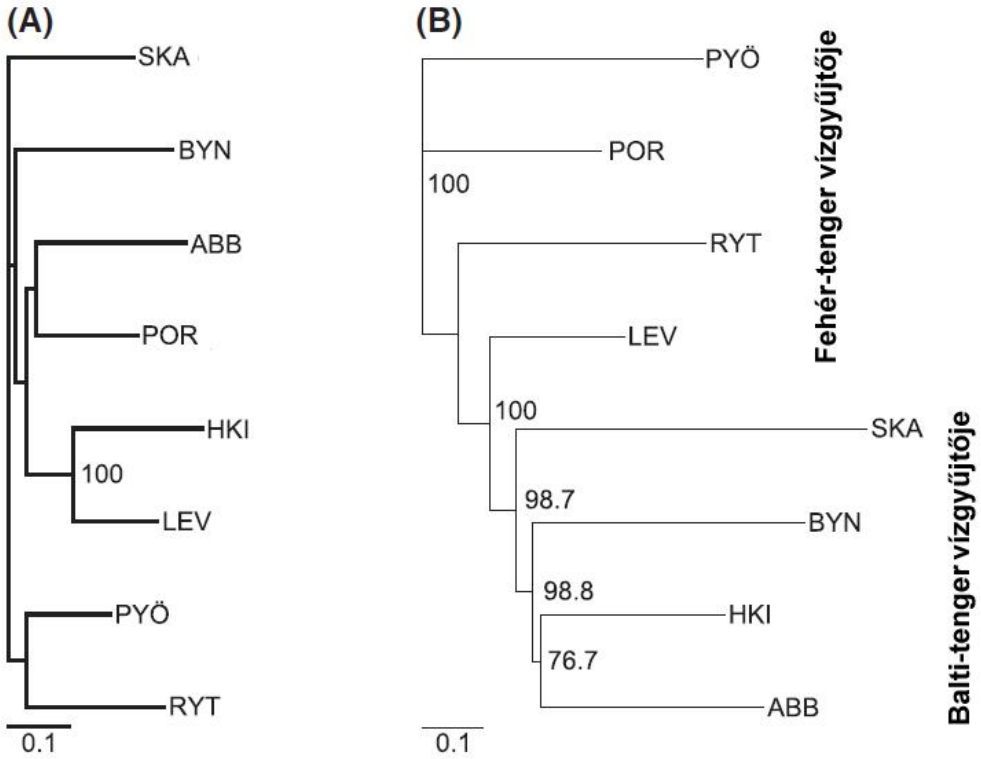
Az északi elterjedésű fajok populációgenetikai struktúráját nagyban meghatározza a déli refúgiumokból történt posztglaciális expanzió, ahol az új élőhelyeket gyakran csak kevés egyed foglalta el, ami a genetikai diverzitás graduális csökkenéséhez vezetett (Hewitt 1996, 1999). A genetikai strukturáltságra hasonlóan fontos hatással bír a jelenlegi élőhely típusa a génáramlás befolyásolásán keresztül. Például a tengeri halpopulációk általában nagy populációmérettel és a populációk között akár jelentős távolságot is legyőző erős génáramlással jellemezhetőek, ami gyenge genetikai strukturáltsághoz vezet, viszont ugyanezen fajok édesvízi populációi a fizikai és egyéb barriereknek köszönhetően markáns genetikai elkülönülést mutatnak (Gyllensten 1985; Ward et al. 1994; DeWoody & Avise 2000). Kelet-Ázsiában a tengerek partvidékén és a belső területek édesvizeiben élő kilenctüskés pikók genetikai elkülönülése olyan erős, hogy gyakorlatilag külön fajokról beszélhetünk (Takata et al. 1987). Ezzel szemben az európai populációk genetikai mintázatairól szinte semmi információ nem állt rendelkezésünkre.



5. ábra. Az európai kilenctüskés pikók (50 populáció) leszármazási viszonyai 12 variábilis mikroszatellita lókusz alapján (módszer: *neighbour-joining*). A populációs kódok első két betűjének jelentése: JP = Japán, DK = Dánia, UK = Egyesült Királyság, NO = Norvégia, BE = Belgium, FR = Franciaország, FI = Finnország, SE = Svédország, EE = Észtország, RU = Oroszország. A kódok második tagja a konkrét mintavételi helyet jelöli (Függelék: Shikano et al. 2010 Molecular Ecology).

A kilenctüskés pikó észak-európai filogeográfiájának és populációgenetikai viszonyainak a feltárásához 25 populációból származó 96 egyed mitochondriális DNS szekvenciáját (citokróm b) és 50 populációból több mint 1700 egyed 11 mikroszatellita és egy beillesztés-törlés (*insertion/deletion*) lókuszát analizáltuk (Shikano et al. 2010c). Mindkét adatsoron ugyanazt az eredményt kaptuk a posztglaciális rekolonizáció és a jelenleg megfigyelhető genetikai leszármazási vonalak tekintetében (5. ábra): jól elkülönült egy nyugati (Norvégia déli csücske és az attól délre eső területek) és egy keleti leszármazási vonal (az összes Fennoskandináv minta). A tengeri populációk genetikai diverzitása magasabb volt, mint az édesvízieké és az édesvízi populációknál a genetikai diverzitás észak felé csökkent. A populációk közötti genetikai differencia átlagos mértéke nagyon magas volt (F_{ST} [a populációk közötti és a teljes neutrális genetikai variancia arányából

számolt index] = 0,415; standard hiba = 0,031). A várakozásainknak megfelelően a tengeri populációk esetében az elkülönülés mértéke jóval alacsonyabb volt, mint az édesvízi populációknál.



6. ábra. Nyolc, a dolgozatban összefoglalt vizsgálatok szemponjából kulcsfontosságú kilenctüskés pikó populáció genetikai viszonyai. Jól látható, hogy amíg a 12 variábilis mikroszatellita lókusz alapján kapott fa (módszer: *neighbour-joining*) kaotikus képet mutat (A), a 3624 polimorfikus RAD marker (Függelék: Bruneaux et al. 2013 Molecular Ecology) már tisztán elkülöníti a Balti- és Fehér-tenger vízgyűjtőit (B). A populációs kódok megtalálhatóak az 1. táblázatban egy kivétellel: a jelen ábra HKI populációja az 1. táblázatban és a 3. ábrán HEL kódon szerepel.

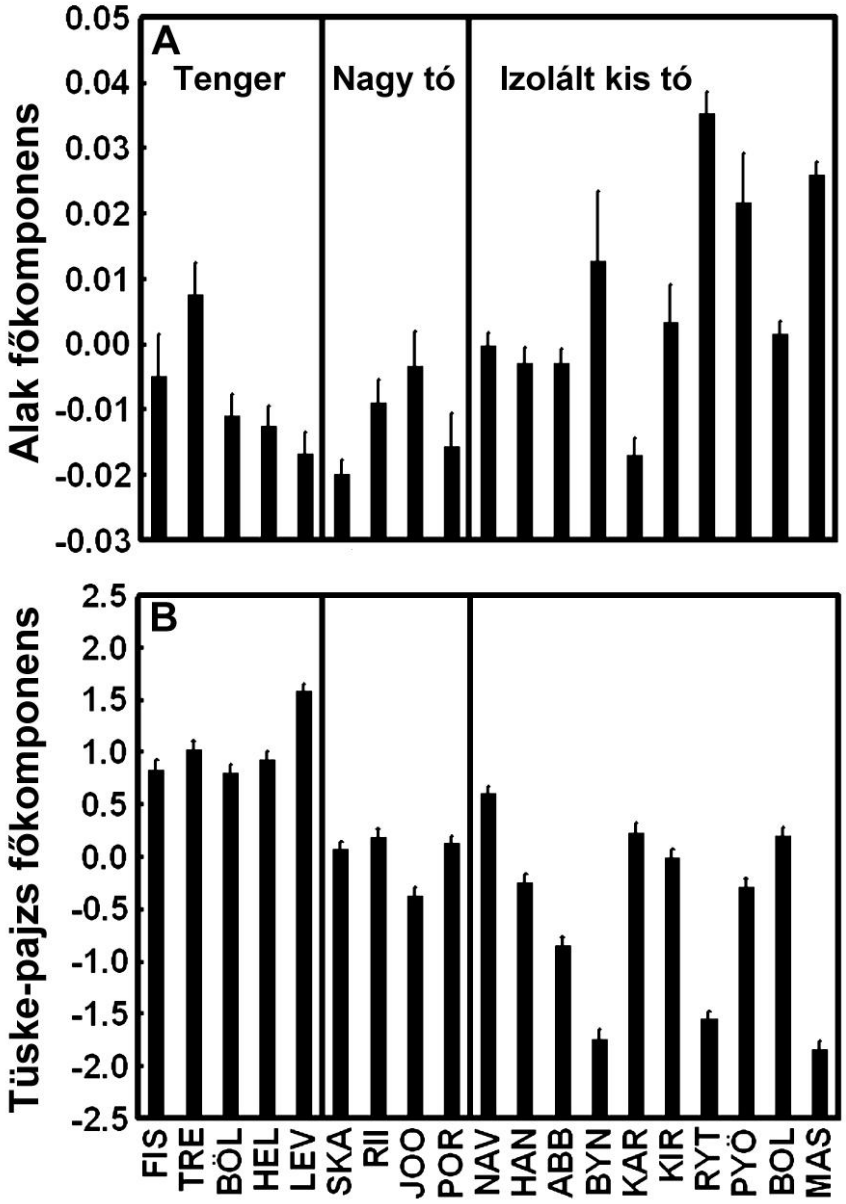
Ezen eredményeink alapján elmondható, hogy a fennoskandináviai kilenctüskés pikó jó modell a populációs összehasonlításokon alapuló evolúciós ökológiai kutatásokhoz mivel (i) a vizsgált populációk egy evolúciós leszármazási vonalba tartoznak bármilyen evolúciós vagy taxonómiai szintű strukturáltság nélkül és (ii) a populációk közötti genetikai eltérés magas, azaz a habitatokon (főleg az édesvízi populációknál) belül a populációkat tekinthetjük független ismétlésnek. Az észak felé csökkenő genetikai diverzitás bizonyításával elsőként támogattunk egy, a posztglaciális rekolonizáció témakörébe tartozó általános hipotézist (Hewitt 1996, 1999; madár: Merilä et al. 1997; kétéltű: Palo et al. 2004) édesvízi halfajon. Egy későbbi lényegesen átfogóbb genomikai vizsgálatunk (Bruneaux et al. 2013; részletesebben lásd 4.3.2.1) finomította a képet, genetikailag elkülönítve a Balti- és a Fehér-tenger vízgyűjtő területét (6. ábra). Tehát ez alapján az alább tárgyalt populációs összehasonlításokban van egy vízgyűjtő terület alapú ismétlés is, tovább erősítve a mintázatok általánosíthatóságát.

4.1.2 Morfológiai különbségek

A predáció az egyik legnagyobb környezeti szelekciós erő, ami a préda szinte minden tulajdonságára hatással lehet (Roff 1992; Stearns 1992). Ezért nem meglepő a nagyszámú ragadozó ellenes tulajdonság evolúciója a legkülönbözőbb taxonómiai szinteken (Tollrian & Harwell 1999). Talán a legismertebb ragadozóellenes tulajdonságok közé tartoznak a morfológiai adaptációk, mint például a sérülésektől óvó kemény felületek, pajzsok, vagy a lenyelés-manipuláció ellen védő tüskék. A morfológiai antipredátor adaptációk egyik tankönyvi példája a háromtüskés pikó, ahol az ősi pelágikus tengeri formák robosztus „fegyverzettel" (pajzsok és tüskék) bírnak, míg a leszármazott édesvízi populációknál ez a fegyverzet gyakran (ismételt módon és független esetekben) erősen redukálódott (Bell & Foster 1994). A fegyverzet szerepe bizonyított: a hasi és háti tüskék a közöttük lévő pajzsokkal merev egységet képezve a lenyelés ellen védik az állatot (Reimchen 1983; Leinonen et al. 2011), a pajzsok pedig a fogakkal rendelkező ragadozók okozta sérülések ellen védenek (Reimchen 1992, 1994). Ugyanez a fegyverzet megtalálható a kilenctüskés pikónál is, gyengébb, de még működő formában (Hoogland et al. 1957). A pajzsok és háti tüskék számában, valamint a hasi tüskék méretében megfigyelt változatosságot a kilenctüskés pikónál már régen leírták mind Észak-Amerikából (McPhail 1963), mind Európából (Gross 1979), de alapos evolúciós vizsgálatnak még nem vetették alá ezeket a tulajdonságokat.

Egy másik klasszikus morfológiai tulajdonság, aminek evolúcióját sok halfajon vizsgálták a testalak. A háromtüskés pikó alakjának evolúciója alaposan kutatott (pl. Reimchen et al. 1985; Walker 1997, Walker & Bell 2000; Leinonen et al. 2006): az ősi pelágikus forma a karcsú, áramvonalas test hosszú faroknyéllel, a leszármazott bentikus forma pedig a robosztus, mély test rövid faroknyéllel. Az ok a szétválásra egyszerű, a pelágikus forma a pelágikus környezetben fontos lassabb, de folyamatos úszást, a bentikus forma pedig a bentikus környezetben fontos gyors megindulást, nagy végsebességet és manőverező képességet segíti (Walker 1997; Bergstrom 2002; Walker et al. 2005). A kilenctüskés pikók alak-változatosságának részletes analízise még váratott magára.

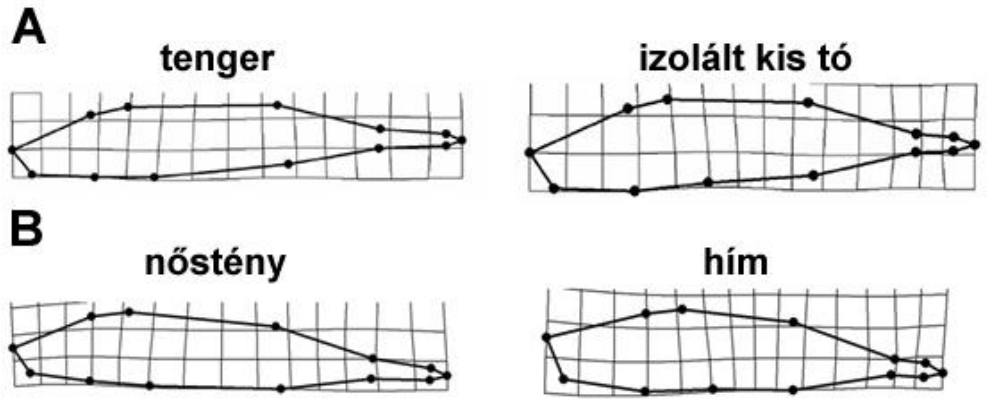
Öt tengeri, négy nagy tavi és 10 izolált kis tavi populációból származó minta alapján feltártuk a Fennoskandináv kilenctüskés pikók csontos fegyverzetének és alakjának változatosságát, két-két független tengeri és izolált kis tavi populáción alapuló *common garden* adatokat is bevonva (Herczeg et al. 2010a). Ezek az adatok kiválóak mind a paralel evolúció vizsgálatára a nagyszámú független kis tavi populációnak az ősinek tekinthető tengeri és nagy tavi populációkhoz való hasonlításával, mind a konvergens evolúció tesztelésére az itt megfigyelt mintázatoknak a háromtüskés pikónál leírtakkal való összevetésével. Emellett feltártuk a kilenctüskés pikó morfológiájában megfigyelhető ivari dimorfizmust is (Turtiainen, Herczeg, Merilä kézirat).



7. ábra. Morfológiai változatosság 19 kilenctüskés pikó populációban (átlag ± standard hiba). A) Változatosság testalakban. Az iránypont-alapú geometriai morfometria analízis eredményeként kapott főkomponens egy gradienst ír le a keskeny, áramvonalas testű és hosszú faroknyelű pikóktól (alacsony értékek) a robosztus, mély testű és rövid faroknyelű pikókig (magas értékek). A testalakok grafikus ábrázolását lásd a 8. ábrán. B) Változatosság a csontos fegyverzetben (hasi tüske, mell-öv és pajzsok). Az itt ábrázolt főkomponens az erősen redukált fegyverzettől (alacsony értékek) a maximálisan kifejezett fegyverzetig (magas értékek) terjedő gradienst írja le. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

Az eredményeink alapján az izolált kis tavi pikók kisebb tüskékkel (néhol hiányoztak a tüskék) és kevesebb pajzzsal voltak felvértezve, valamint mélyebb, robosztusabb testtel és rövidebb faroknyéllel bírtak, mint a tengeri fajtársaik (7. ábra). A nagy tavak pikói a tengeri fenotípushoz álltak közel. A habitatokon belül, főleg az izolált kis tavaknál, meglehetősen nagy változatosság volt a populációk között. A *common garden* eredmények mindenben támogatták a természetből gyűjtött mintákon megfigyelt mintázatokat. Az ivari dimorfizmus sokban hasonlított

a tenger – izolált kis tó szétválásra: a hímek teste mélyebb, robosztusabb volt, mint a nőstényeké, és a faroknyelük is rövidebb volt (8. ábra). Sőt, a hímek tüskéi rövidebbek, a pajzsaik száma pedig alacsonyabb volt, mint a nőstényeké.



8. ábra. Kilenctüskés pikók testalak-változatossága. A) Élőhely-alapú szétválás. B) Ivari dimorfizmus. Az változatosság optimális megjelenítése érdekében mindkét esetben az extrém fenotípusokat mutatom be.

A morfológiai változatosság habitat-függésének, valamint a *common garden* eredmények és a vizsgált karakterek pikóknál ismert heritabilitása (Hagen 1973; Blouw & Boyd 1992; Hermida et al. 2002; Leinonen et al. 2010) alapján feltételezhető genetikai hátterének figyelembe vételével valószínűsíthető a természetes szelekció által létrehozott paralel evolúció (Clarke 1975; Endler 1986; Schluter & Nagel 1995; McGuigan et al. 2005). Bár a nagy tavi pikók testalak tekintetében teljesen a tengeri fajtársaikra hasonlítottak, a csontos fegyverzet terén már inkább a tengeri és a kis izolált tavi fenotípusok között helyezkedtek el (7. ábra). A pikók fegyverzetének redukciójára két fő elképzelés van (Reimchen 1994; Boughman 2007, Huntingford & Coyle 2007). Az első hipotézis a ragadozókkal függ össze. Az ízeltlábú ragadozók (pl. szitakötőlárvák) a fegyverzet ellen szelektálnak, mivel könnyebben meg tudják tartani a tüskés-pajzsos halakat. A ragadozó halak nyílt vízben a kifejezett fegyverzet irányában (más védelem nem nagyon van), bentikus környezetben pedig a fegyverzet ellen szelektálnak (fegyverzet nélkül gyorsabbak és fordulékonyabbak a halak, könnyebben el tudnak rejtőzni) (Marchinko 2009; Leinonen et al. 2011). A második hipotézis szerint a vízben oldott, csontépítéshez elengedhetetlen ionok mennyisége a tengervízben magasabb, mint az édesvízben, és ez okozza az édesvízben gyakran megfigyelt fegyverzet-redukciót (Giles 1983; Bell et al. 1993; Marchinko & Schluter 2007). A mi esetünkben mindkét hatás elképzelhető, a tenger és nagy tó élőhelyek közötti eltérést az ion különbség magyarázhatja, a nagy tó és kis izolált tó közötti különbségre pedig jó magyarázat a ragadozó halak hiánya és az ízeltlábú ragadozók nagy száma az utóbbi élőhelyen.

Mind a csontos fegyverzet, mind a testalak tekintetében a háromtüskés pikóknál leirt mintázatokhoz hasonló eredményeket kaptunk, ami a több mint 10 millió éve szétvált (Shapiro et al. 2006; Bell et al. 2009; Aldenhoven et al. 2010) fajpárt a

konvergens evolúció klasszikus példájává emeli. Mivel a háromtüskés pikó esetében a szétválás többé-kevésbé pontos genetikai háttere is ismert (Peichel et al. 2001; Shapiro et al. 2004; Colosimo et al. 2005; Miller et al. 2007; Chan et al. 2010), egy remek modellünk van tesztelni, hogy a konvergens fenotípusok mögött azonos vagy eltérő gének állnak (lásd 4.3.2.2.1).

A megfigyelt ivari dimorfizmus nagy valószínűséggel a pikóknál általánosan eltérő ivari szerepekből és a hozzájuk tartozó eltérő viselkedési mintázatokból ered. A hímek a fészeképítésnek és –őrzésnek, valamint a territorialitásuknak és ivadékgondozásuknak köszönhetően az év nagy részében bentikusabb életmódot követnek, mint a rajban járó és a hímek fészkeihez csak ikráikat lerakni járó nőstények (Wooton 1984; Kitano et al. 2007). Ez egyszerűen magyarázza a hím – nőstény eltérés feltűnő hasonlóságát az izolált kis tó – tenger (vagy nagy tó) eltéréshez. Persze a fenotípusok szétválása is kényszerek alatt áll. Leinonen et al. (2010) bebizonyította, hogy a fegyverzet és testalak között genetikai korreláció áll fenn a háromtüskés pikónál, azaz ezek a tulajdonságok nem tudnak függetlenül evolválódni. Amennyiben a genetikai korreláció a kilenctüskés pikónál is megvan, akár eltérő szelekciós erők is eredményezhetnek hasonló fenotípusos különbségeket a habitatok illetve az ivarok között.

4.1.3 Életmenetbeli eltérések

4.1.3.1 Testméret, növekedés, ivarérés időzítése

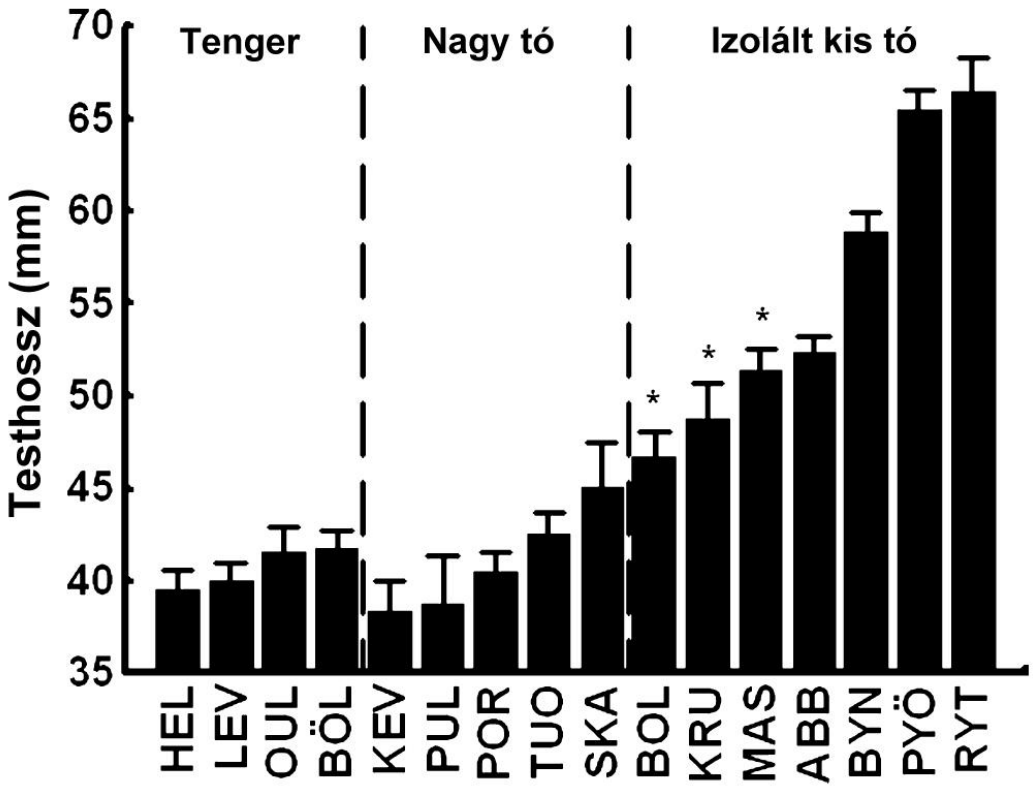
A testméret egy kiemelt fontosságú tulajdonság az ökológia és evolúcióbiológia területén, mivel gyakran befolyásol fontos élettani tulajdonságokat és hatással bír a rátermettségre (Peters 1983; Roff 1992; Stearns 1992). Az ivari szelekció különböző formái (párválasztás, nemen belüli verseny) és a fekunditási szelekció általában a nagyobb méretet részesíti előnyben (pl. Shine 1989; Andersson 1994; Arendt 2010). Sőt, a természetes szelekcióban is előnyt jelenthet a nagy méret, például ha a nagyobb egyedet a méret-limitált ragadozó már nem veszélyezteti (Werner & Gilliam 1984; Abrams & Rowe 1996). Makroevolúciós léptékben ismert és bizonyított a Cope-szabály, miszerint egy adott evolúciós leszármazási ágban az átlagos testméret idővel nő (Cope 1887; Alroy 1988). Mégis, számtalan esetben nem figyelhetünk meg növekedést sem mikro- sem makroevolúciós kontextusban. Mi lehet ennek az oka? Erre a kérdésre kereste Blanckenhorn (2000) a választ, és mutatott rá a predáció szerepére, ugyanis a méret által nem limitált ragadozók (ez a gyakori eset) kimondottan preferálják egy adott prédafajon belül a nagyobb egyedeket. Egy másik közismert ok az interspecifikus kompetíció: a forrásokért való versengésen alapuló közösségben a részt vevő fajok méretváltozása limitált (Wilson 1975; Lomolino 1985). Ezeket az elméleteket nem könnyű fajon belül tesztelni, hiszen ritka helyzet az, ahol a populációk között tiszta jelenlét-hiány eltéréseket találunk predációban vagy interspecifikus kompetícióban. Egy klasszikus lehetőség a sziget – kontinens összehasonlítás; az előbbi élőhelyeken gyakran drasztikusan leegyszerűsödött közösségeket találunk ahol a predáció és interspecifikus kompetíció

evolúciós szerepe lecsökken, nem véletlenül nevezte Mayr (1967) az ilyen rendszereket természetes kísérletnek.

Nem csak a nagy kifejlett méret, hanem a gyors növekedés is sok szempontból előnyös: például több idő marad a szaporodásra és a méret-limitált ragadozók elöl is hamarabb szabadul a gyorsabban növő egyed (Sibly & Calow 1986; Abrams & Rowe 1996; Blanckenhorn & Demont 2004). Ennek ellenére sok esetben figyelhetünk meg az élettanilag maximálisnál lassabb növekedést a természetben (Calow 1982; Atchley 1984). A gyors növekedés ellen ható két fő faktor a forrás-hiány, és az aktív táplálékkeresés okozta fokozott kitettség a ragadozóknak (Lima & Dill 1990; Abrams & Rowe 1996; Day & Rowe 2002; Biro et al. 2004, 2006). Érdekes módon, amíg a földrajzi gradiens menti populációs növekedési ráta összehasonlítások gyakoriak, az eltérő predációs nyomás alapú összehasonlítások ritkák (Dmitriew 2011). A növekedési stratégiától nehezen elválasztható ivarérési életkor szintén nagy hatással bír a rátermettségre (Roff 1992; Stearns 1992). Általában a lehető legkorábbi ivarérés az előnyös, különösen nagy predációs nyomás alatt (Stearns 1992). Mindazonáltal, a szaporodás – túlélés vagy jelen szaporodás – jövőbeli szaporodás negatív kapcsolata (*trade-off*) miatt speciális esetekben az ivarérés kora kitolódhat (Reznick et al. 1990; Roff 1992, 2002; Stearns 1992). Az ivarérés korának predációs nyomás-függő kitolódására aránylag kevés példa van (pl. Reznick & Ghalambor 2005).

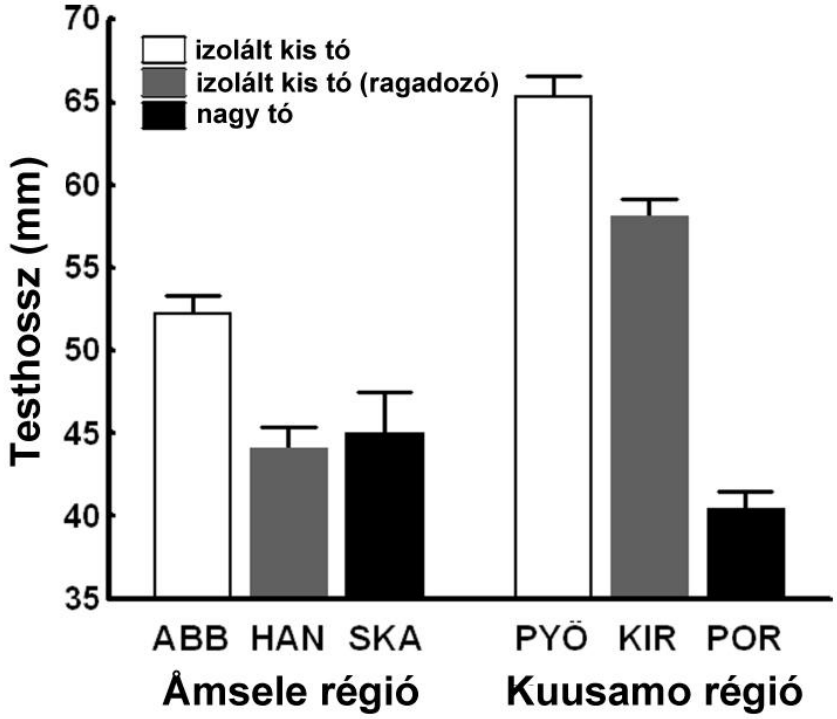
Hipotézisünk szerint ahol egy pikópopuláció a nagy testméret és gyors növekedés ellen ható ragadozók és a méretváltozások ellen ható más fajú versenytársak nélkül szabadon evolválódhatott, óriás testméretet kell megfigyelnünk. Az óriás méret eléréséhez pedig a növekedési időszak elnyúlása kapcsolódik, akár az ivarérés idejének kitolása árán is.

A természetes populációkban a kifejlett kilenctüskés pikók méretét négy tengeri, öt nagy tavi és kilenc izolált kis tavi populáció mintáinak felhasználásával vetettük össze (Herczeg et al. 2009a). Az izolált kis tavak között volt olyan ahol a kilenctüskés pikó volt az egyetlen halfaj, volt ahol a háromtüskés pikóval, és volt ahol a ragadozó sebes pisztránggal (*Salmo trutta*) együtt fordult elő, így vizsgálni tudtuk a predáció és interspecifikus kompetíció és a testméret kapcsolatait. A növekedési stratégiákat összevetettük egyrészt egy természetből származó kisebb minta (két tengeri, egy nagy tavi és négy izolált kis tavi populáció) korának meghatározása után (Herczeg et al. 2009a), másrészt egy két független tengeri és két független izolált kis tavi populáción alapuló *common garden* kísérlet adatainak felhasználásával (Herczeg et al. 2012). Az ivaréres idejét egy másik, egy tengeri és egy izolált kis tavi populáción végzett *common garden* kísérletből becsültük (Ab Ghani et al. 2013). Ez utóbbi kísérlet tulajdonképpen a populációs szétválás kvantitatív genetikai hátterének vizsgálatához (4.3.1) lett végrehajtva, de a tiszta populációs vonalak eredményei idevágnak.

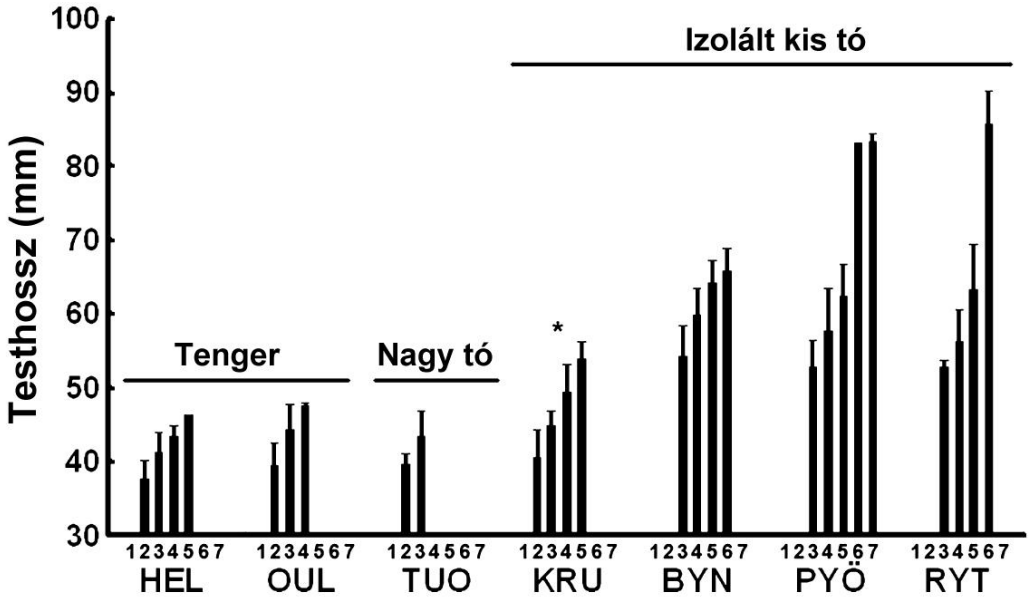


9. ábra. Kilenctüskés pikók testméret-változatossága (átlag + 95% konfidencia intervallum). A * jel azokat az izolált kis tavi populációkat jelöli, ahol a kilenctüskés pikók mellett háromtüskés pikók is előfordulnak. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

A természetben megfigyelt mintázatok megfeleltek a várakozásainknak (Herczeg et al. 2009a): amíg a tengeri és nagy tavi populációkban a kilenctüskés pikók a szakirodalomban (Bănărescu & Paepke 2001) általánosan ismertetett testhosszal bírtak, az izolált kis tavakból származó pikók sokkal nagyobbak voltak (1. & 9. ábrák). Amennyiben az izolált kis tavakat szigeteknek tekintjük, a vizsgálatot felfoghatjuk a Sziget-szabály intraspecifikus tesztjének. A Sziget-szabály (Foster 1964; Van Valen 1973a,b; Lomolino 1985, 2005) azt mondja ki, hogy szigetszerű élőhelyeken az apró termetű fajok nagyobbra, az óriás termetű fajok pedig kisebbre evolválódnak. A méretváltozások fő hajtóerejének a predációs nyomás és a kompetíciós viszonyok változását tartják. Az eredményeink támogatják a Sziget-szabályból levont fajon belüli predikciókat, a ragadozó és kompetitor halfajok hiányában a testméret lényegesen nagyobb. Ez utóbbiak szerepét az adataink támogatták, ahol az izolált kis tavakban jelen volt ragadozó vagy kompetitor halfaj is, ott a pikók testmérete a tengeri – nagy tavi fajtársaikéhoz közelített (9 & 10. ábrák).



10. ábra. Ragadozó betelepítés hatása az izolált kis tavi kilenctüskés pikók testméretére (átlag + 95% konfidencia intervallum) két geográfiailag és genetikailag is távoli régióban. Mindkét esetben sebes pisztráng (*Salmo trutta*) volt a betelepített halfaj. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.



11. ábra. Életkor-függő testméret-változatosság (átlag + 95% konfidencia intervallum) különböző kilenctüskés pikó populációkban. A * jel azt az izolált kis tavi populációt jelöli, ahol a kilenctüskés pikók mellett háromtüskés pikók is előfordulnak. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

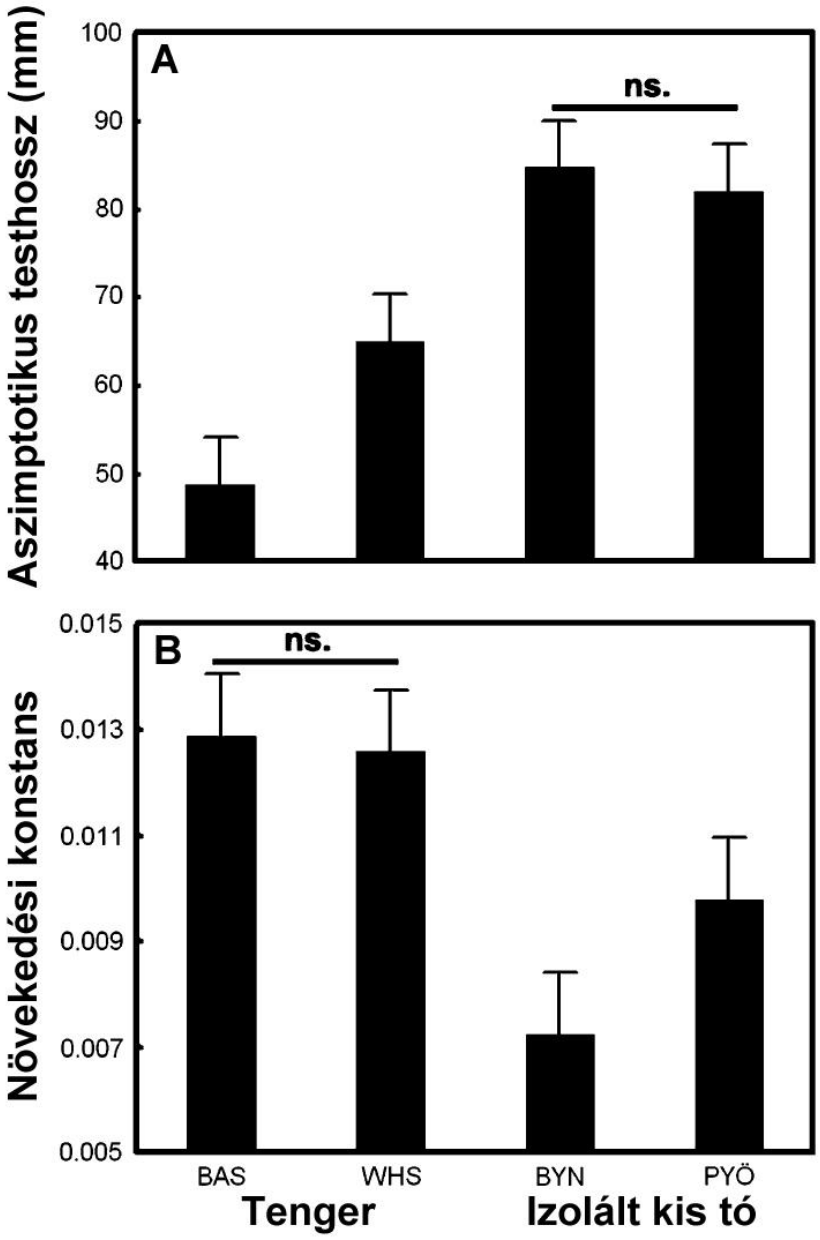
A méretbeli különbségeket két – egymást nem kizáró – mechanizmus is okozhatta, a ragadozók hiányában megnövekedett élettartam és a magasabb növekedési ráta. Az adataink alapján mindkettő jelen van a rendszerben, az izolált kis tavi pikók jóval tovább élnek, mint a tengeri vagy nagy tavi fajtársaik, de azonos életkoron belül is egyértelműen nagyobbak (11. ábra). Persze ez alapján még nem tudható, hogy a különbség genetikailag meghatározott vagy fenotipikus plaszticitás eredménye, amely egyedül is jelentős méretkülönbségeket eredményezhet (Madsen & Shine 1993). A növekedési stratégiák *common garden* kísérletből származó adatokon való analízise azonban világosan mutatta a szétválást (Herczeg et al. 2012). Az izolált kis tavakból származó pikók becsült végleges mérete jelentősen meghaladta a tengeri pikókét, míg a növekedési konstansuk (a végleges méret elérésének sebességét becsli) a tengeri pikókénál jelentősen alacsonyabb volt (12. ábra). Jól elkülönült tehát a két stratégia, gyorsan kicsivé fejlődni (tenger) vagy lassan óriássá (izolált kis tó). Az utóbbi esetben a lassú fejlődés az időre vonatkozik, hiszen az izolált kis tavakból származó pikók egységnyi idő alatt nagyobb testméret-gyarapodást mutattak. A két vizsgált növekedési stratégiát leíró változó negatív korrelációt mutatott, ami általános jelenség (pl. Berrigan & Charnow 1994) és könnyen értelmezhető egy élemenet *trade-off*-ként (Roff 1992; Stearns 1992).

A Fehér-tengerből származó pikók – bár stratégiájuk tekintetében tisztán elkülönültek az izolált kis tavi halaktól – becsült végső testmérete jelentősen meghaladta a Balti-tengerből származókét (12. ábra). Különösen érdekes ez, mivel a vadon befogott szülői generációk a két tengerből azonos méretűek voltak (9. ábra; Herczeg et al. 2009a). Gyakorlatilag a növekedési stratégiákat célzó *common garden* kísérlet (Herczeg et al. 2012) végére a laborban felnőtt fehér-tengeri halak körülbelül 2 cm-rel (50%) nagyobbak voltak, mint az akkor még a laborban szintén életben lévő vadbefogott szülői generációjuk tagjai. Ez a mintázat a *countergradient variation* (Conover & Schultz 1995) példájának tűnik, ahol a genetikai potenciál és a realizált fenotípus nincs összhangban egymással, mert a genetikai adaptáció hatását a természetben elnyomják a direkt környezeti hatások (jelen esetben a rövid aktivitási időszak). Ez a feltételezés további vizsgálatokat érdemel.

Végezetül azt találtuk, hogy az izolált kis tavakból származó pikók később érik el ivarérettségüket, mint a tengeri fajtársaik (Ab Ghani et al. 2013). A hímek esetében ez egyszerűen csak időeltolódást jelentett, az izolált kis tavi nőstények viszont egy telelés után egyáltalán nem kerültek szaporodási kondícióba. Ez utóbb mintázatot erősítik a természetből gyűjtött szaporodó egyedek koreloszlása (11. ábra; Herczeg et al. 2009a) és a több éves laboratóriumi megfigyeléseim is (lásd még 4.3.1.2).

Ezek az eredmények egyértelműen támogatják azt a hipotézisünket, miszerint a predációs nyomás extrém csökkenésekor, amennyiben domináns kompetitor fajok nem fordulnak elő, óriás testméret evolválódik elnyújtott növekedési időszakkal és késleltetett ivaréréssel. Ennek fő oka az intraspecifikus verseny előtérbe kerülése, ahol mind a különböző forrásokért való harcban, mind pedig a fekunditást illetően a

sikert a nagy méret hozhatja el. A megfigyelt, majd kétszeres élettartam az izolált kis tavi populációknál sejteti, hogy az ivarérés kitolásával nem sokat vesztenek az állatok, az óriás nőstények megnövekedett fekunditása (4.1.3.2; Herczeg et al. 2010b; Ab Ghani et al. 2012) pedig kimondottan az óriás testméret elérésének rátermettség előnyeit mutatja be.



12. ábra. Populációs szétválás a kilenctüskés pikók növekedési stratégiáiban a von Bertelanffy féle növekedési modell két paraméterén keresztül szemléltetve. A) Aszimptotikus testhossz és B) növekedési konstans (átlag + 95% konfidencia intervallum). Az „ns." az adott élőhelyen belüli összehasonlításoknál a szignifikáns eltérés hiányát jelöli.

Kevés hasonló mintázatról találni beszámolót az irodalomban, talán a leginkább idevágó eredményeket szivárványos guppikon (*Poecilia reticulata*) végzett vizsgálatok adták. A magas predációs nyomással jellemezhető élőhelyeken korai

ivarérést és nagyobb szaporodási befektetést figyeltek meg (Reznick 1982; Reznick & Endler 1982) jelentős méretbeli eltérés nélkül (Hendry et al. 2006). Ez a rendszer azonban különbözött a mienktől annyiban, hogy a ragadozó halak okozta magas és alacsony predációs nyomással jellemezhető élőhelyek között a ragadozók okozta mortalitás korfüggésében volt eltérés. A magas predációs nyomású élőhelyeken kortól (és mérettől) független volt a ragadozók hatása, míg az alacsony predációs nyomású élőhelyeken a ragadozók főleg a fiatalabb (kisebb), ivaréretlen guppi korosztályt fogyasztották (Reznick et al. 1996). Érdekességként megemlítem a háromtüskés pikónál megfigyelt gigantizmust is, ami a kilenctüskés pikóval szemben éppen a fajjal együtt élő ragadozóknak köszönhető (Moodie 1972a,b; Moodie & Reimchen 1976; Reimchen 1988, 1991). Az eltérés a háromtüskés pikóval együtt élő ragadozó méret-limitáltságában rejlik, ami miatt a ragadozó direkt módon a pikók gyors növekedésére és óriás méretére szelektál.

Az itt tárgyalt populációs mintázatok valószínűsíthető genetikai meghatározottsága és habitat-specificitása egyértelműen a természetes szelekció munkájára utal (Clarke 1975; Endler 1986; Schluter & Nagel 1995; McGuigan et al. 2005). A természetes szelekcióban fontos feltételezett környezeti faktorokat, úgymint a predációt, az interspecifikus kompetíciót, és az előbbiek csökkenésekor előtérbe kerülő intraspecifikus kompetíciót is megneveztük. Az elképzelésünk közvetlen bizonyítása azonban nem könnyű. A legbiztosabb módszer az adott tulajdonságok heritabilitását és genetikai korrelációit becslő, illetve a különböző feltételezett szelekciós erőket manipuláló kvantitatív genetikai kísérletsorozat lett volna, de ehhez nem voltak meg a lehetőségeink. Ezért egy másik oldalról, statisztikai modellezéssel közelítettük a problémát, és teszteltük a fent említett biotikus környezeti faktorok szerepét a méret- és növekedés szétválásában (Aikio et al. 2013). A modellező megközelítés persze nem adhat olyan bizonyító erőt, mint a kísérletes adhatott volna, de támogató eredmények esetén a hipotézisünket megerősítheti.

A modell a von Bertalanffy növekedesi görbén (von Bertalanffy 1938) és a növekedési konstans és a becsült maximális méret közötti negatív korreláción (Charnov 1993; Berrigan & Charnow 1994) alapult. Lényegében különböző átlagos ragadozó okozta mortalitások mellett modelleztük az optimális növekedési stratégiát, figyelembe véve a (i) növekedés intenzitásának emelkedésével járó fokozott aktivitást, ami viszont a ragadozóval való végzetes találkozás esélyét növelte, (ii) a késleltetett ivarérésből származó rátermettség eltéréseket és (iii) a fekunditás – testméret összefüggést. A modelleket a Herczeg et al. (2012) vizsgálat valós adataival parametrizáltuk.

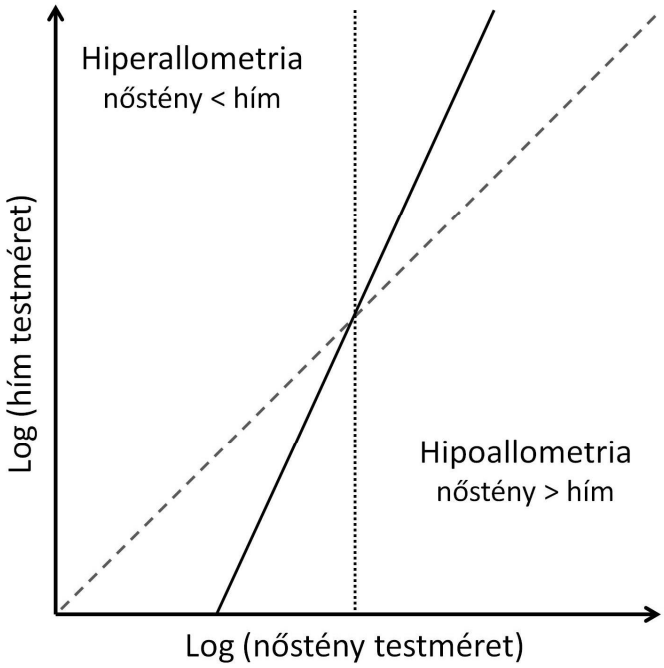
A modellek eredményei teljes egészében támogatták a hipotézisünket (Aikio et al. 2013). Magas ragadozó okozta mortalitásnál a rátermettség növelésének fő lehetősége a legalább egy szaporodási eseményben való részvétel volt „bármi áron”. Magyarán a halaknak ilyen környezetben a lehető leghamarabb el kell érni az élettani és törzsfejlődési kényszerek által kialakított minimális szaporodó testméretet és a lehető leghamarabb szaporodniuk kell. Amennyiben viszont a ragadozók hatása

jelentéktelen, a halak jó eséllyel élnek sokáig, több szaporodási eseményben véve részt. A rátermettségüket ilyenkor meghatározó komponens a méret-függő fekunditás, és ezért az élettani és törzsfejlődési kényszerek által kialakított maximális testméret elérése a cél. A teljes élet alatti szaporodási siker (*lifetime reproductive success*) mértéke olyan erős negatív kapcsolatban van az ivarérés idejével, hogy eleinte szinte elképzelhetetlennek tartották a késleltetett ivarérés evolúcióját (Cole 1954). Később ez az álláspont megváltozott, és a jelenséget markáns fekunditási vagy túlélési előny esetén már reálisnak tartották (Charnov & Schaffer 1973; Bulmer 1994). Több potenciális mechanizmus is részt vehet a késleltetett ivarérés előnyösségében. A korai ivarérésnek önmagában is vannak nagy költségei: erősen csökkenti az egyed végső testméretét (Charnov 1993; Berrigan & Charnow 1994) és túlélését (Bell 1980; Kuparinen et al. 2012). A nagyobb testméret ugyanakkor megnöveli a sikert a kompetíciós szituációkból adódó direkt konfrontációban és növeli a fekunditást (Peters 1983; Roff 1992; Stearns 1992). Ennek ellenére az ivarérés késleltetése csak extrém körülmények között valósulhat meg, a ragadozóktól független mortalitásnak alacsonynak kell lennie, a ragadozóktól függő mortalitásnak viszont szinte a nullához kell tartania (Aikio et al. 2013). Az általunk vizsgált izolált kis tavakban a kilenctüskés pikó számára ezek a feltételek teljesülnek, és így az általunk megfigyelt mintázatok értelmet nyernek.

4.1.3.2 Ivari méretdimorfizmus és fekunditás

Az átlagos méreten kívül a nemek közötti méretkülönbség (ivari méretdimorfizmus) is variálhat populációk között. Az ivari (méret)dimorfizmus több, egymást nem kizáró okra is visszavezethető. A főbb elképzelések szerint a különböző nemi szerepekhez kapcsolódó szexuális szelekció, vagy a tisztán ökológiai faktorok által meghatározott nemek közti verseny eredményezhet fenotípusos szétválást egy populációban a hímek és nőstények között (pl. Shine 1989; Andersson 1994). Az ivari méretdimorfizmus mind az állatok, mind a növények között általános (Fairbairn 1997). Gerinctelen és ektoterm gerinces állatoknál legtöbbször a nőstény, endotermeknél pedig a hím a nagyobb (Fairbairn 1997; Blanckenhorn 2005). Ahogy a populációk közötti általános eltérés a szelekciós erőkben általános fenotípusos szétválást okozhat, ugyanúgy eredményezhet a változatosság az ivar-specifikus szelekciós erőkben populációs szétválást az ivarok közötti eltérések mértékében. Régi megfigyelés, hogy az ivari méretdimorfizmus mértéke összefügg az összehasonlított fajok vagy populációk átlagos méretével: ahol a nőstény a nagyobb ott a mérettel csökken (hipoallometria), ahol pedig a hím a nagyobb ott a mérettel nő (hiperallometria) az ivari méretdimorfizmus mérteke. Ezt a jelenséget a leírójáról Rensch-szabálynak nevezték el (Rensch 1950, 1959; Fairbairn 1997). Ha a hipo- és hiperallometriát képzeletben összekötjük, akkor egy folyamatos változást kapunk ahol a fajok vagy populációk méretváltozásakor a hímek testmérete nagyobb mértékben változik, mint a nőstényeké (13. ábra). Ez alapján azt feltételezik, hogy a Rensch-szabály mögött a hímekre ható szexuális szelekció áll (pl. Berry & Shine 1980; Székely et al. 2004; Serrano-Meneses et al. 2009). A Rensch-szabályt sok

vizsgálat támogatta, főleg ott ahol a hímek nagyobbak, de az inverz Rensch-szabályra is vannak példák, főleg ott ahol a nőstények a nagyobbak (Fairbairn 1997).

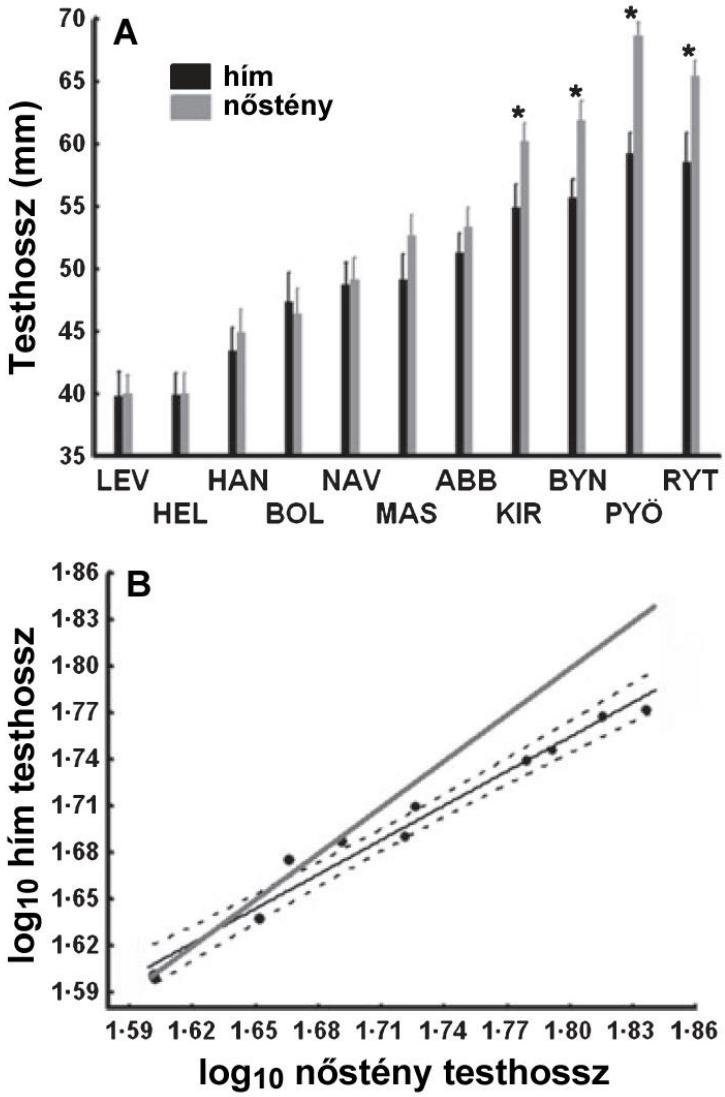


13. ábra. A Rensch-szabály: az ivari méretdimorfizmus foka a vizsgált taxon (populáció) átlagos méretétől függ. Ha a nőstények a nagyobbik nem, akkor a taxonok (populációk) méretének növekedtével az ivari méretdimorfizmus csökken (hipoallometria), ha a hímek a nagyobbak, akkor viszont az ivari méretdimorfizmus a taxonok (populációk) méretének növekedtével nő (hiperallometria). A szürke szaggatott vonal mutatja az izometriát (nőstény méret = hím méret), a fekete vonal egy Rensch-szabálynak megfelelő összefüggést, míg a vízszintes pontozott vonal választja el a hipo- és hiperallometrikus szakaszt. Az ábra Abouheif és Fairbairn (1994) 1. ábrájának módosított változata.

A Rensch-szabály talán legszélesebb körben elfogadott potenciális mechanizmusa a korreláló szelekció (*correlational selection*) (Fairbairn 1997). Ez alapján az ivari méretdimorfizmusnál megfigyelhető allometria az egyik nemre ható direkcionális szelekció (pl. szexuális szelekció a hímeknél vagy fekunditási szelekció a nőstényeknél) eredménye, az ebből eredő és a másik nemre ható korreláló szelekcióval kombinálva. Persze elképzelhető, hogy mindkét nemre hat méret-függő szelekció akár azonos, akár ellentétes irányban, de az egyik nemre ható szelekció dominál, és így meghatározza a populáció változásának irányát. Mi a korreláló szelekció hipotézist, mint keretrendszert használtuk annak az eldöntésére, hogy a kilenctüskés pikóknál a hímekre vagy a nőstényekre ható szelekciónak volt döntő hatása a fajnál megfigyelt extrém méretváltozásokra.

Két független tengeri és kilenc izolált kis tavi populáció ivari méretdimorfizmusának analízisét végeztük el (Herczeg et al. 2010b). A mintánk lefedte a fajnál ismert teljes mérettartományt, és azokra a populációkra szorítkozott ahol elegendő mintaszámunk volt mindkét nem ivarérett egyedeiből. A Rensch-szabály inverzére kaptunk bizonyítékot: a populációkra jellemző átlagos testmérettel

párhuzamosan nőtt az ivari méretdimorfizmus mérteke, holott a nőstények nagyobbak voltak, mint a hímek (14. ábra). Ezt a mintázatot egy kisebb *common garden* vizsgálatból eredő adatsor is támogatta.



14. ábra. A) Ivari méretdimorfizmus 11 kilenctüskés pikó populációban (átlag + 95% konfidencia intervallum), lefedve a faj teljes ismert méretspektrumát. A * jel azokat a populációkat jelöli, ahol szignifikánsan eltért a nemek mérete. B) Az ivari méretdimorfizmus allometriája: a populációk átlagos méretével nő az ivari méretdimorfizmus mértéke, holott a nőstény a nagyobb nem (a Rensch-szabály inverze).

Normál testméretű populációkon belül ismert volt a nőstények fekunditásának pozitív méret-függése (Heins et al. 2003, 2005), de mi is összehasonlítottuk a *common garden* kísérletekbe vont nőstények fekunditását méretük és származási helyük alapján (Herczeg et al. 2010b). A peték száma drasztikusan eltért a tengeri és izolált kis tavi nőstények között, a különbség elérte háromszorost (tenger < kis tó), és a peték mérete is hasonló, de nem szignifikáns trendet mutatott. A frissen kikelt, önállóan még nem táplálkozott halak testméretében is lényeges különbség mutatkozott (tenger < kis tó). Két populáció alaposabb analízisével populáció-

független pozitív lineáris mérethatást találtunk a peteszám-peteméret és a nőstények mérete között (Ab Ghani et al. 2012).

Az eredményeinket a korreláló szelekció elmélet fényében értelmezve elmondhatjuk, hogy a fajnál megfigyelt extrém méretkülönbségek (Herczeg et al. 2009a, 2012) javarészt a nőstényekre ható fekunditási szelekció eredményei. Feltehetőleg a hímek szempontjából is előnyös a nagy testméret, hiszen territoriális fajról van szó, de döntőnek az ikracsomónként 2-3-szor nagyobb szaporodási teljesítmény látszik, amit az óriás nőstények produkálnak a normál méretűekhez képest. Ezek alapján a főleg nőstényeknél szembeszökő késleltetett ivarérés (Ab Ghani et al. 2013) is logikusnak látszik, az akár hét évig is elélő nőstények (Herczeg et al. 2009a) nem kockáztatják a korai ivaréréssel járó testméret-csökkenést (Charnov 1993; Berrigan & Charnow 1994). Sőt, a tény, hogy a növekedési stratégia (növekedési konstans, becsült végső méret) tekintetében is főleg az izolált kis tóból származó nőstények mutattak markáns eltérést a tengeri fajtársaiktól (Herczeg et al. 2012) is értelmet nyert.

4.1.3.3 Energiaraktárak

Az energia felvétele, raktározása és felhasználása az életmenet szempontjából kulcsfontosságú folyamatok. Az energia raktározásának fő célja, hogy az állat sikeresen túléljen és akár szaporodjon olyan időszakokban, amikor a táplálkozása limitált (McNamara & Houston 1990; Varpe et al. 2009). Noha az energia raktározásának vannak költségei (Jönsson 1997), ektotermeknél ezek a költségek általában elhanyagolhatók (Bonnet et al. 1998). Alacsonyabb rendű gerinceseknél az energia általában zsírok és szénhidrátok formájában tárolódik az izomzatban, a testüregben található zsírtestben és a májban (Fitzpatrick 1976; Chellappa et al. 1989, 1995). Az energia energiaraktárakba való fektetése gyakran *trade-off* viszonyban áll a növekedéssel és a szaporodási befektetéssel, és ezért az életmenet-stratégiák szerves része (Roff 1992; Stearns 1992). Sőt, az energia raktározása direkt módon is kapcsolatba állhat az predációs veszéllyel (Veasey et al. 1998; Pérez-Tris et al. 2004). Ismervén az eddig taglalt életmenet-stratégia szétválást a kilenctüskés pikó populációi között, felmerül a kérdés, hogy milyen energiaraktározási stratégiát követnek az izolált kis tavi és a tengeri populációk? A hipotézisünk szerint optimális körülmények között (táplálék *ad libitum*, ragadozók nincsenek) a kis tavi pikók minden energiát a növekedésbe fektetnek minimális raktározás mellett a versenyhez való adaptációjuk miatt, a tengeri pikók viszont igyekeznek annyit raktározni, amennyit csak lehet, felkészülvén az esetlegesen következő veszélyes időszakra.

A kérdésünkre egy, elsődlegesen a fenotipikus plaszticitás kérdéskörének (4.2) vizsgálatára tervezett, négy tengeri és két izolált kis tavi kilenctüskés pikó populáció bevonásával kivitelezett *common garden* kísérletünk (Välimäki, Herczeg, Merilä kézirat) kontroll csoportjai adtak választ. Az eredményeink összetettek voltak. A kis tavi pikók relatíve nagyobb belső szerv-mentes testtömeggel, viszont kisebb zsírtesttel és májjal rendelkeztek, mint tengeri fajtársaik. A nemeknek is volt hatása,

a hímek relatíve nagyobb belső szerv-mentes testtömeggel, viszont kisebb májjal rendelkeztek, mint a nőstények (a részletes mintázatokért, beleértve a kezeléseinket is, lásd: 4.2.1.2.2).

A belső szerv-mentes testtömegnél talált különbségek magyarázata nem egyértelmű. Ez a változó egyaránt reprezentálhatja a funkcionális szövetekbe (izomzat és csont) és a raktározásba (lipidek és glikogén) való invesztálást (Chellapa et al 1989; Huntingford et al. 2001). A nagyobb izomtömeg növelheti a versenyképességet (Casselman & Schulte-Hostedde 2004; Stahlschmidt et al. 2011), ami a kis tavi pikóknak fontos lehet a feltételezett magas intraspecifikus kompetíció miatt. Az erős kompetíció jelenlétét támogatják a később (4.1.5, 4.2.1.4) tárgyalt viselkedéshez kötődő eredményeink is (Herczeg et al. 2009b,c, Herczeg & Välimäki 2011). Ugyanakkor az izomtömeg a menekülést is elősegítheti (Domenici et al. 2008). Bár minden jel a kompetíciós elképzelést támogatja, további célzott kísérletek nélkül az ellenkezője sem zárható ki.

A zsírtest és májtömeg mintázatokat ellenben könnyű interpretálni. Sok esetben találták azt, hogy nagyobb halak több energiát raktároznak a kisebbeknél (Schultz & Conover 1997; Robards et al. 1999; Sogard & Spencer 2004), de nálunk nem ez volt a helyzet. Ahogy prediktáltuk, a növekedésre koncentráló nagyobb testű kis tavi pikók nem raktároztak, a túlélésre játszó tengeri pikók viszont a helyzetet kihasználva sok energiát fektettek a raktáraikba. Összességében az eredményeink jól illettek az eddig felállított elképzelésünkbe az életmenet-stratégiák szétválásáról. A lehető legnagyobb testméretre törekvő izolált kis tavi pikók feltehetően a strukturális szöveteikbe fektetik az energiát, hiszen a szaporodási sikerük elsősorban a testméretüktől, versenyképességük pedig az izomtömegüktől függ. A túlélésre és gyors ivarérésre/szaporodásra alkalmazkodott tengeri pikók viszont a veszélytelen és táplálékban gazdag környezetet kihasználva, a raktáraikba fektetik az energiát, mivel az ő szaporodási sikerük főleg az első szaporodási időszakig való túlélésen alapul.

4.1.4. Agy és érzékszervek

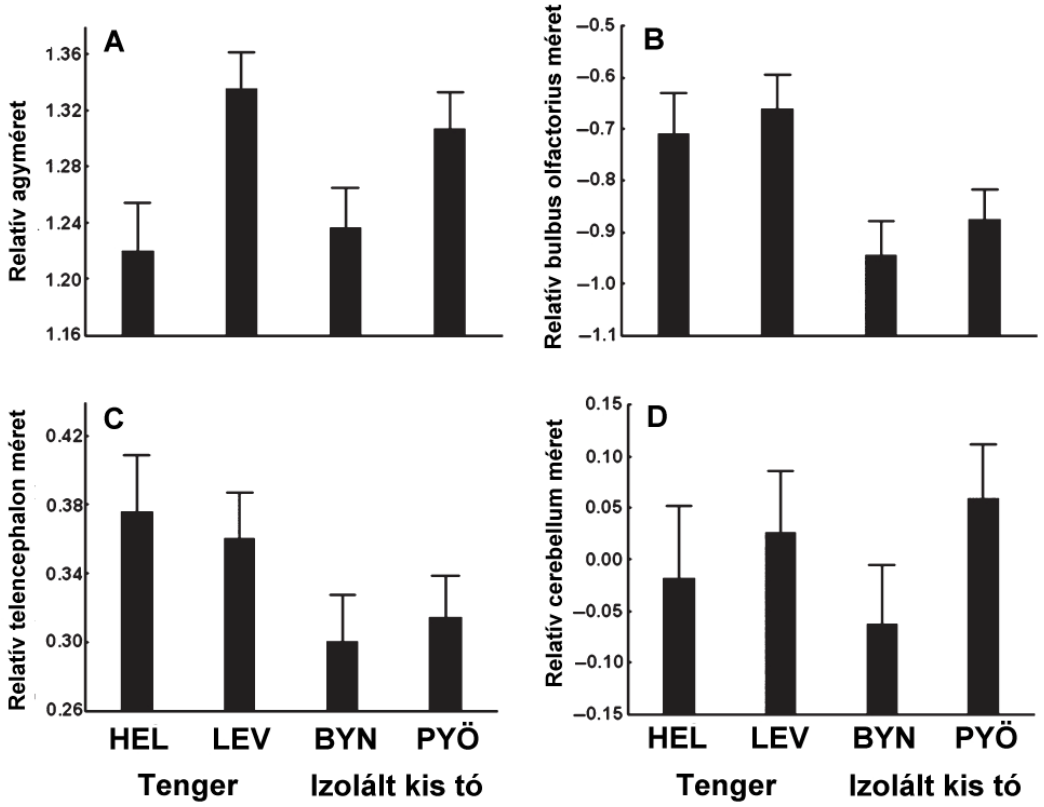
4.1.4.1 Agyméret és az agyterületek mérete

Az agy mindig kiemelt érdeklődést kapott a biológia szinte minden állatokkal foglalkozó területén, köszönhetően az egyed és környezete közötti kapcsolatokban elfoglalt központi szerepének. Dacára az agykutatásban használt módszertani diverzitásnak, az evolúciós ökológiában (nem utolsó sorban a vizsgált fajok és a szükséges nagy mintaszámok miatt) az agyméret és az agy főbb részeinek mérete a változók, melyekkel az agynak és területeinek fejlettségét becslik (Striedter 2005). Mivel az idegszövetnek a fejlesztése és fenntartása egyaránt rendkívül költséges (Aiello & Wheeler 1995; Kotrschal et al. 2013), az energetikai kényszerek erős szelekciós erőt képviselnek a feleslegesen nagy agy ellen. Ezért az agy vagy valamely agyterület mérete az adott képlet fontosságának jó mérőszáma (de Winter & Oxnard 2001; Gonzalez-Voyer & Kolm 2010).

Az agy evolúciójának kutatása két fő pilléren nyugszik: (i) a fajok vagy magasabb taxonok közötti filogenetikailag kontrollált összehasonlítások (pl. Aviles & Garamszegi 2007; Pollen et al. 2007; Gonzalez-Voyer et al. 2009; Barton & Capellini 2012; Fitzpatrick et al. 2012, van Woerden et al. 2012) és (ii) a populáción belüli fenotipikus plaszticitásra koncentráló vizsgálatok a gyakoriak (Maguire et al. 2000; Tramontin & Brenowitz 2000; Kihslinger & Nevitt 2006; Kihslinger et al. 2006; Lisney et al. 2007; Draganski & May 2008). A két pillér között helyezkedik a sokáig mellőzött fajon belüli, populációk összehasonlításán alapuló mikroevolúciós megközelítés, ami sok szempontból ígéretesebb a többinél (Gonda et al. 2013). A fajon belüli evolúciós megközelítés előnyei számosak, például (i) a populációk gyakran abban a környezetben találhatóak, amihez genetikailag alkalmazkodtak, az (ii) adaptív és neutrális szétválás elkülöníthető, (iii) bármely kvantitatív tulajdonság evolúciójának megértéséhez szükséges főbb paraméterek (egyedek közötti változatosság, heritabilitás, genetikai korrelációk, szelekciós erők) kísérletesen becsülhetők, sőt, az (iv) egyedi változatosság mögötti gének azonosíthatók (Gonda et al. 2013). Azonban egyelőre még nagyon kevés az ilyen vizsgálat.

A kilenctüskés pikó rendszerben gondolkodva több dolgot is figyelembe vehetünk. Egyrészt az izolált kis tavi pikókról bebizonyosodott, hogy a ragadozó nyomás alól kiszabadulva redukálták a költséges csontos védelmi képleteiket, óriási méretre nőnek, és raktárakat nem képezve mindent a növekedésbe fektetnek. Másreszt igen egyszerű, ingerszegény környezetben élnek. Az izolált kis tavakban nincs másik halfaj, a víztestek kicsik, és az általunk vizsgált kis tavak strukturális komplexitása is igen alacsony volt (minimális mennyiségű vízinövény, pár kő, vízbe esett faág). Ezzel szemben a tengeri vagy nagy tavi pikók védelmi képletei kifejezettek, végső méretük kicsi, ha tudnak, raktároznak. Ráadásul egy strukturálisan összetettebb környezetben élnek nagyszámú ragadozó és versenytárs halfajjal együtt, ahol gyakran még migrációs viselkedést (íváskor felúsznak kis patakokba) is mutatnak. Mindezek alapján a tengeri pikóknál relatíve nagyobb agyat, illetve nagyobb memóriáért, tanulásért és érzékelésért felelős agyterületet feltételeztünk, mint a kis tavi pikóknál.

Először egy két tengeri – két izolált kis tavi populáción alapuló *common garden* kísérletből származó mintákat hasonlítottunk össze (Gonda et al. 2009a). Az agytérfogat, valamint a *telencephalon*, a *bulbus olfactorius* és a *cerebellum* relatív mérete tért el a populációk között (15. ábra). A négy képlet közül a *telencephalon* és a *bulbus olfactorius* mutatott habitat-specificitást: mindkét esetben a tengeri pikóknak volt relatíve nagyobb agyterülete, mint a kis tavi pikóknak.

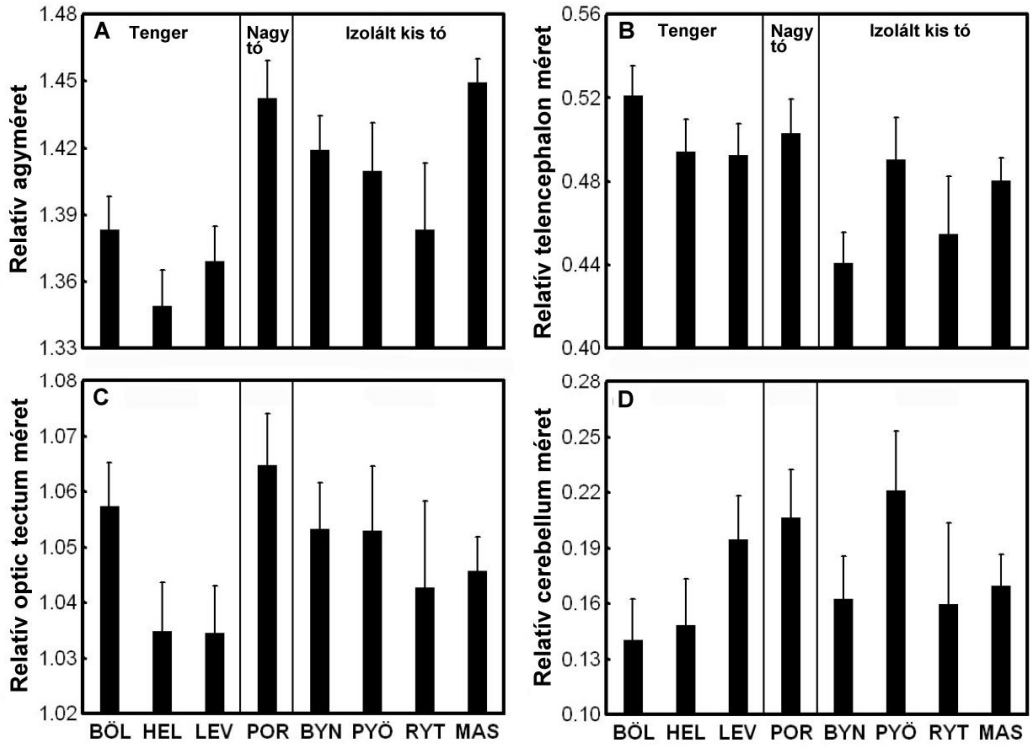


15. ábra. Populációs szétválás agyméretben és a különböző agyterületek méretében (átlag + 95% konfidencia intervallum) kilenctüskés pikók első laboratóriumi generációjánál. A) Agyméret, B) *bulbus olfactorius* mérete, C) *telencephalon* mérete, D) *cerebellum* mérete. Az ábrán $\log_{10}$-transzformált adatok szerepelnek testméretre, és az agyterületek esetén az agyméretre is korrigálva. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

Az eredményeink két fő pontban foglalhatóak össze. Először is bizonyítottunk fajon belüli populációs szétválást az agyméretben és a különböző agyterületek méretében. Másodszor, bizonyos agyterületeknél a populációs szétválás habitat-specifikus volt. Elfogadva, hogy a genetikailag megalapozott habitat-függő populációs mintázat természetes szelekciót és adaptív szétválást jelez (Clarke 1975; Endler 1986; Schluter & Nagel 1995; McGuigan et al. 2005), mi voltunk az elsők, akik természetes populációk vizsgálatával bizonyítottak fajon belüli adaptív agy evolúciót. Mivel a vizsgálati területeink körülbelül 8000 évvel ezelőtt még jéggel voltak borítva (Eronen et al. 2001), a mintázatok gyors evolúcióra utalnak. A *telencephalon* és a *bulbus olfactorius* esetében a hipotézisünkkel egybevágó eredményt kaptunk, a leegyszerűsödött környezetben élő, és energiájukat főleg növekedésbe fektető kis tavi pikóknál ezek az agyterületek relatíve kisebbek voltak, mint a tengeri pikóknál.

A *telencephalon* mérete halaknál pozitívan korrelál a habitat komplexitásával (Huber et al. 1997). *Telencephalon*-írtott halak tanulási, habituációs és elkerülési képességei szignifikánsan csökkentek (Laming & McKinley 1990; Portavella et al. 2003). Anatómiai vizsgálatok szerint a *telencephalon* részt vesz az emocionális és térbeli tanulásban (Broglio et al. 2003). Ezeket az eredményeket figyelembe véve

logikusnak tűnik a következtetés miszerint az élőhely biotikus és abiotikus komplexitásának és a tanulás fontosságának csökkenése állhat az izolált kis tavi pikók *telencephalon* csökkenése mögött. A *bulbus olfactorius* az agy szaglóközpontja (Gonda 2011). Hogy pontosan milyen okból nagyobb a tengeri pikók ezen agyterülete, csak találgatni tudunk. Elképzelhető például, hogy a tengeri környezetben rosszabbak a látási viszonyok, mint a kis tavakban, és ezért a szaglásnak kiemelt szerep jut. Ez az érzékelési forma fontos lehet a ragadozó halak elkerülésében is. A térbeli orientációban, motoros koordinációban és szem-mozgások irányításában fontos szereppel bíró *cerebellum*-nál (Kotrschal et al. 1998) megfigyelt mintázatot (az egyik kis tóban kisebb, mint a többi vizsgált populációban) jelenleg nem tudjuk magyarázni. Az agyméretben megfigyelt változatosságot magyarázhatja a különböző vízgyűjtő területekhez tartozás és az ehhez kapcsolódó genetikai differentáció (Bruneaux et al. 2013). Szisztematikus környezeti eltérésről nem tudunk a vízgyűjtő területek között, de akár törzsfejlődési kényszerek is elképzelhetőek.



16. ábra. Populációs szétválás agyméretben és a különböző agyterületek méretében (átlag ± standard hiba) vadbefogott kilenctüskés pikóknál. A) Agyméret, B) *telencephalon* mérete, C) *tectum opticum* mérete, D) *cerebellum* mérete. Az ábrán $\log_{10}$-transzformált adatok szerepelnek testméretre, és az agyterületek esetén az agyméretre is korrigálva. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

A *common garden* kísérlet után három tengeri, egy nagy tavi és négy izolált kis tavi populációt is összehasonlítottunk, ezúttal a természetből gyűjtött kifejlett pikók felhasználásával (Gonda et al. 2011). A természetben megfigyelhető mintázatok több ponton is eltértek a *common garden* kísérletben megfigyelttől. Az agyméret,

valamint a *telencephalon*, az *tectum opticum* és a *cerebellum* mérete is eltért a populációk között (16. ábra). A *bulbus olfactorius*-t ezeken a mintákon nem tudtuk analizálni. A *telencephalon*-nál kapott mintázat megegyezett a *common garden* kísérletben (Gonda et al. 2009a) látottakkal, azaz a tengeri pikóknál ez az agyterület nagyobb volt, mint a kis tavi pikóknál. Az agyméret azonban, mind abszolút, mind relatív mértékben nagyobbnak bizonyult a kis tavi pikóknál, mint a tengeri fajtársaiknál. A többi változónál megfigyelt változatosság nem mutatott habitat-specificitást. A két megközelítés eredményei között tapasztalt eltérések megértése érdekében elvégeztünk egy analízist azokon a populációkon ahol mind a természetből, mind a *common garden* kísérletből volt adatunk. Az összes vizsgált változóra hatással volt a mintavételi módszer. Laborkörülmények között az összes agyterület relatíve kisebbre fejlődött, mint a természetben. Az agyméret ráadásul habitat-függő hatást mutatott: a kis tavi pikók agya kisebb volt a laborban, mint a természetben, a tengeri pikóknál viszont ez a hatás nem volt kimutatható.

Talán a legmeghökkentőbb eredmény a kis tavi pikóknak a tengerieknél nagyobb agya volt. Az abszolút méretet tekintve ez logikus. Ismeretes az egyszerű összefüggés, miszerint a nagyobb testhez a hatékonyság megtartása mellett nagyobb agy is kell (Striedter 2005). Mivel a kis tavi pikók óriássá evolválódtak (Herczeg et al. 2009a, 2012) nem meglepő, hogy az agyuk is nagyobb lett. Annál meglepőbb, és a hipotézisünknek is ellentmond a kis tavi pikók tengerieknél nagyobb relatív agymérete. A természetből gyűjtött mintákon végzett fajon belüli agy-összehasonlítások, ha ritkák is, de ismertek. A kanadai cinege (*Poecile atricapillus*) *hippocampus*-ának mérete és a benne lévő neuronok száma korrelál a környezet „kíméletlenségével" (Roth & Pravosudov 2009). Egy másik madárfaj, a koronás levélsármány (*Zonotrichia leucophrys*) migráns és nem vonuló alfajainak összehasonlításakor a migráló alfajnál találtak nagyobb *hippocampus*-t és több *hippocampus* neuront (Pravosudov et al. 2006). A sebes pisztráng szimpatrikus, migráns és az édesvizet el nem hagyó szaporodási stratégiát követő egyedei között is kimutattak különbségeket agyméretben és a *cerebellum* méretében (Kolm et al. 2009). Ezeknek a vizsgálatoknak az eredményét a szerzők evolúciós szempontból, adaptív szétválásként interpretálták, holott nem volt módjuk a direkt környezeti indukciót, azaz a fenotipikus plaszticitást tesztelni, vagy kizárni. Az eredményeink rávilágítanak ennek a megközelítésnek a sebezhetőségére. A mi esetünkben is sikerült habitat-függő eltéréseket kimutatni a természetből gyűjtött mintákból mind az agyméret, mind a telencephalon méret tekintetében. Mindkét esetet adaptív evolúciós különbségként lehetne interpretálni, ha nem tudnánk, hogy a standardizált *common garden* körülmények között az agyméretnél nyoma sincs a mintázatnak. Tehát a vadon befogott pikóknál észlelt agyméret mintázat a fenotipikus plaszticitás valamelyik formájának és nem az adaptív evolúciónak az eredménye, míg a telencephalon mintázatnál valószínűsíthető az adaptív evolúció. Ez nagyon fontos különbség, amivel kapcsolatban egyéb tulajdonságoknál többen is megerősítették a kontrollált környezet fontosságát a fenotípusból közvetlen evolúciós következtetést levonni kívánó vizsgálatoknál (Gienapp et al. 2008; Alho et al. 2010). A halak

neurogenezise egész életük alatt folytatódik, és így életük minden szakaszában képesek lehetnek plaszticitásra (Birse et al. 1980; Zupanc 2001, 2006), amelyre az evolúciós agy vizsgálatoknál mindenképpen kontrollálni kell.

Az eredményeink másik fontos vetülete az, hogy a relatív agyméret és az agystruktúra eltér egy adott populáció természetből gyűjtött és laboratóriumban nevelt egyedei között. Sőt, az eltérés az agyméretnél habitat-specificitást mutatott. A háziasítás negatív hatása az agyméretre jól ismert, a mintázatot a genetikai adaptáció és a fenotipikus plaszticitás közösen okozza (Kruska 1988; Kihslinger et al. 2006). Guppiknál is bizonyítást nyert a laboratóriumi tartás agyméret-csökkentő hatása (Burns et al. 2009). Az általunk talált általános csökkenés egybevág a várakozásokkal, de az agyméret habitat-függő csökkenése meglepő volt, és további vizsgálatokat igényel. Mindenesetre a tény, hogy a természet – *common garden* eltérés akár habitat-függő is lehet, jól mutatja, milyen óvatosnak kell lennünk az evolúciós következtetések levonásánál.

A későbbiekben tárgyalt fenotipikus plaszticitást vizsgáló kísérletünkben (4.2.1.3.1; Gonda et al. 2012; Herczeg et al. 2014) lehetőség nyílt az ivarok összehasonlítására is. A hímek agymérete, valamint *telencephalon*, *cerebellum* és a *hypothalamus* mérete is jelentősen (6-16 %) meghaladta a nőstényekét (Herczeg et al. 2014). Ez magyarázható a faj szaqporodásbiológiájával. A hímek egy sor szaporodási viselkedést mutatnak, ilyen például a territórium őrzése, fészeképítés, násztánc, ivadékgondozás, míg a nőstények szaporodási befektetése főleg a peték létrehozására szorítkozik. A szaporodási viselkedésbe többet fektető ivarnak jobb kognitív képességekre, és következésképpen fejlettebb idegrendszerre van szüksége (Jacobs 1996). A mi eredményeink tökéletesen illeszkednek a képbe, és hasonló ivari dimorfizmust figyeltek meg háromtüskés pikóknál is (Kotrschal et al. 2012). Ugyanakkor az ivari különbségek nem mutattak habitat-függést, tehát az ivari szelekció valószínüleg hasonló a vizsgált élőhelyeken, és a természetes szelekció is hasonló mindkét ivarnál.

4.1.4.2 Oldalvonalszerv

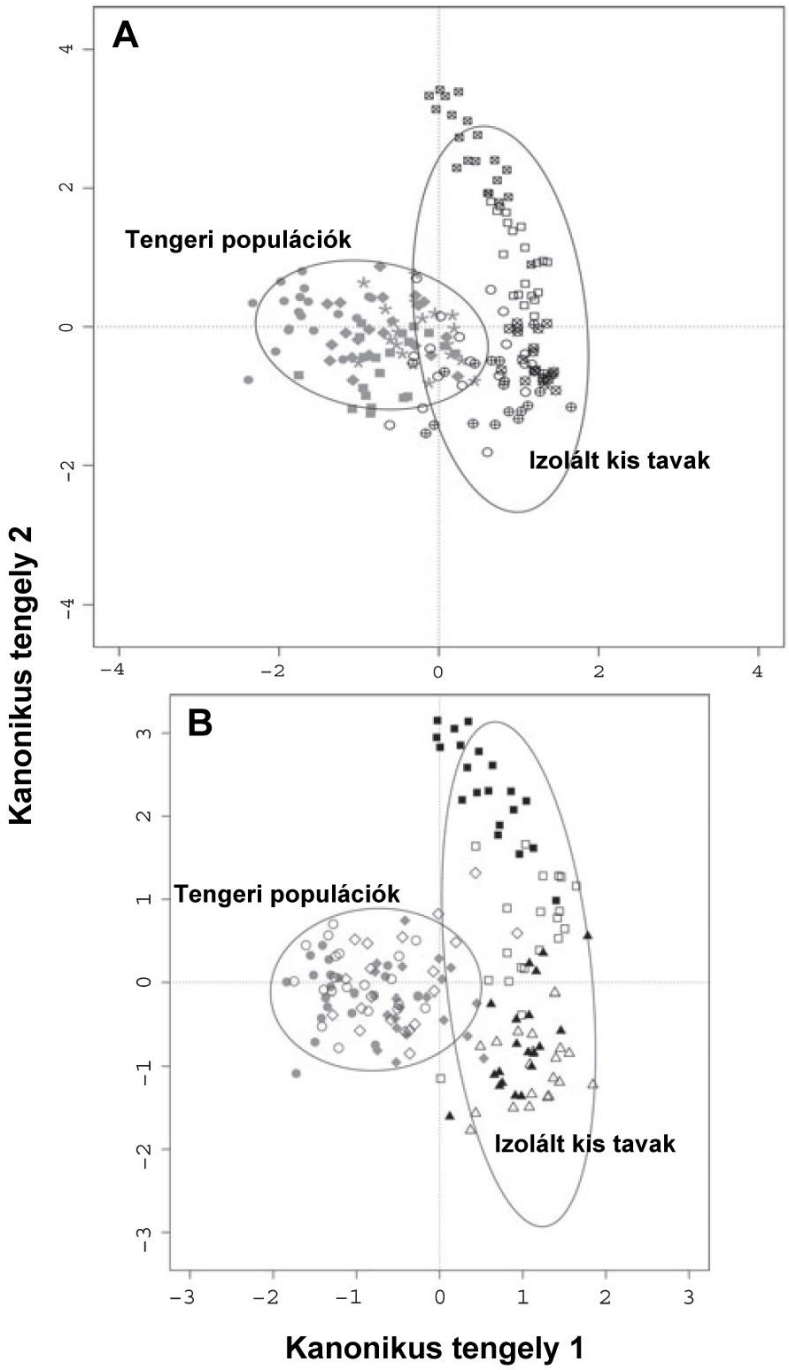
Az oldalvonalszerv a halak és vízi kétéltűek speciális mechanoreceptor-alapú érzékszerve, amellyel a víz áramlásának finom változásait képesek érzékelni (Dijkgraaf 1963). Fontos a ragadozók (Blaxter & Fuiman 1990; Fuiman 1993) és a táplálék (Hoekstra & Janssen 1985; Montgomery & MacDonald 1987) lokalizációjában, valamint a rajképzésben (Partridge & Pitcher 1980). Az oldalvonal funkcionális alapegysége a neuromaszt (érzékelő sejtek csoportja). A neurmomasztok vagy a testfelszínen (felszíni neuromaszt) vagy közvetlenül a testfelszín alatt folyadékkel kitöltött csatornákban (csatorna neuromaszt) találhatóak. A felszíni neuromasztok a vízáramlás sebességére érzékenyek (Van Netten & Kroese 1987; Kalmijn 1988) és így fontosak például a rheotaxisban (Montgomery et al. 1997). A csatornákban lévő neuromasztok viszont a vízáramlás sebességének változásaira érzékenyek (Kalmijn 1988; Engelmann et al. 2000) és fontosak a préda

érzékelésében (Coombs et al. 2000) vagy a rajképzésben (Cahn et al. 1968; Gallego & Heath 1994).

Az oldalvonalszerv neuromaszt struktúrája nagy változatosságot mutat fajok között (Coombs et al. 1988; Webb 1989). A barlangi vagy éjszakai aktivitású fajok relatíve sok neuromaszttal bírnak (Montgomery et al. 2001; Carton & Montgomery 2004). Az állóvizekben élő, lassan úszó fajokra inkább a felszíni neuromasztok, míg a gyorsan úszó, folyóvízi fajokra inkább a csatornákban lévő neuromasztok nagy száma jellemző (Webb 1989; Montgomery et al. 1995; Engelmann et al. 2000). A fajok közötti összehasonlítások nagy száma mellett feltűnő a fajon belüli, populációs összehasonlítások elhanyagoltsága (Michel et al. 2008, Wark & Peichel 2010).

17. ábra. Az oldalvonaszervet alkotó egyes oldalvonalak. A felszíni neuromasztokat fehér pontokkal, a csatornákban lévő neuromasztokat pedig fehér karikákkal jelöltem. Felszíni neuromasztok: nazális (N), mandibuláris (M), infraorbitális (IO), felső mandibuláris-preorbitális (MPrU), alsó mandibuláris-preorbitális (MPrL), poszt-orbitális (PO), pre-operculáris (Pr), fejtetői (DH), otikus (Ot), operkuláris (Op), faroknyéli (CP). Csatorna neuromasztok: szupraorbitális (SO-c), infraorbitális (IO-c), pre-operkuláris (Pr-c), poszt-otikus (Pot-c), törzsi (ATr-c), faroknyéli (CP-c).

Mi négy tengeri és öt izolált kis tavi kilenctüskés pikó populáció oldalvonalszervét hasonlítottuk össze (Trokovic et al. 2011a), a természetből gyűjtött mintákra és 11 felszíni és hat csatornákban lévő oldalvonalra alapozva (17. ábra). Felhasználtunk továbbá két tengeri és két izolált kis tavi populáció *common garden* kísérletből származó mintáit is. Túl az egyszerű összehasonlításokon, megpróbáltuk azonosítani a természetes szelekció alatt álló oldalvonalakat. Ehhez először becsültük a populációk közötti neutrális genetikai divergenciát 23 mikroszatellita marker felhasználásával. Ezt használtuk referenciaként (a random genetikai sodródás által létrehozható divergencia becslése; F_{ST}) az oldalvonalak populációs divergenciájának összehasonlításához (fenotípusos divergencia; P_{ST}). A klasszikus kvantitatív genetikai divergencia (Q_{ST}) – F_{ST} összehasonlítások (Merilä & Crnokrak 2001; a módszert részletesebben ismertetem a 4.1.7 pontnál) analógiájára végeztünk P_{ST} – F_{ST} összehasonlítást (Leinonen et al. 2006). $P_{ST} > F_{ST}$ a szétválasztó, a $P_{ST} < F_{ST}$ a pedig a stabilizáló szelekciót jelzi. Amikor P_{ST} nem különbözik szignifikánsan az F_{ST}-től, nem zárhatjuk ki a random genetikai sodródást.



18. ábra. A tengeri és izolált kis tavi populációk oldalvonaszerv elválása kanonikus korreszpondencia analízis alapján. A) Vadbefogott kilenctüskés pikók. ★= FIS, ■ = BÖL, ◆ = HEL, ● = LEV, ⊠ = BYN, □ = ABB, ⊗ = RYT, ⊕ = PYÖ, ○ = MAS. B) Egyes kilenctüskés pikó populációk vadbefogott egyedei és laboratóriumi generációja együtt ábrázolva. Teli szimbólumok = vadbefogott pikók, üres szimbólumok = laboratóriumi egyedek. Szürke szín = tengeri populáció, fekete szín = izolált kis tó. Kör = LEV, rombusz = HEL, négyzet = BYN, háromszög = PYÖ. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

A sokváltozós analízis tiszta elválást diagnosztizált a tengeri és izolált kis tavi pikók oldalvonalszerve között (18/a. ábra). A tengeri pikók oldalvonalszervében több neuromaszt volt. A *common garden* adatok megerősítették az elválás tényét (18/b.

ábra), az elválás genetikai hátterét valószínűsítve. Ivari dimorfizmusra utaló jeleket nem találtunk. Az egyváltozós analízisek alapján két csatornában lévő és egy felszíni oldalvonal, az ATr-c a CP-c és CP oldalvonalak neuromaszt számában talált változatosság volt a habitat alapú szétválásban a legfontosabb. Sőt, ez volt az a három oldalvonal ahol a P_{ST} meghaladta az F_{ST}-t, jelezve, hogy a szétválás a természetes szelekció eredménye. A tengeri habitaton belüli heterogenitás kisebb volt, mint az izolált kis tavi habitatban megfigyelt. A 17 vizsgált oldalvonalból 11 különbözött a populációk között.

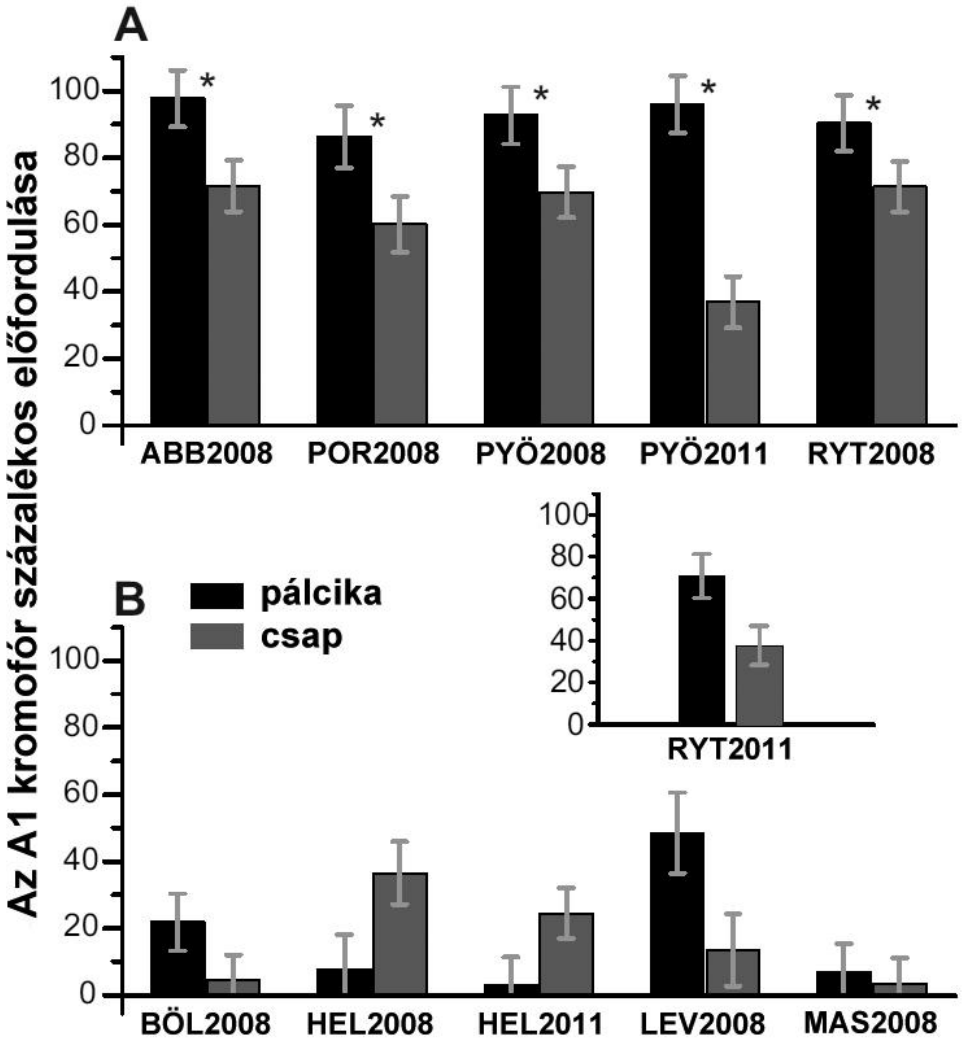
Az érzékszervek fajon belüli változatosságának evolúcióbiológiai vizsgálata még gyerekcipőben jár. A jelen vizsgálatban két nagy gerinces taxonra jellemző speciális érzékszerv esetében bizonyítottunk habitat-specifikus populációs szétválást a természetből gyűjtött és *common garden* mintákon egyaránt, amit a természetes szelekció által irányított adaptív evolúció jeleként értékelhetünk (Clarke 1975; Endler 1986; Schluter & Nagel 1995; McGuigan et al. 2005). Ezen túlmenően, $P_{ST} - F_{ST}$ összehasonlítást alkalmazva, három oldalvonalnál sikerült a szétválasztó természetes szelekciót tetten érni. Bár a P_{ST} csak durva becslése a pedigré információn (azaz kvantitatív genetikai információn) alapuló Q_{ST}-nek (Pujol et al. 2008), a prediktív ereje általában mégis nagy (Leinonen et al. 2008). Összességében tehát a természetes szelekció okozta szétválás a legvalószínűbb. A tengeri habitatnál megfigyelt alacsony heterogenitással jellemezhető magas neuromaszt számot magyarázhatja az oldalvonalszerv tengeri habitaton belüli kiemelt fontossága és a stabilizáló szelekció. Ezzel szemben a kis tavi populációk közötti kevesebb neuromaszt nagy változatossággal inkább a véletlen folyamatokat, mintsem a szétválasztó szelekciót valószínűsíti. A habitatok közötti legfontosabb releváns különbségnek a predációs nyomás tűnik. A predációs nyomás sokféleképpen teheti fontossá a fejlett oldalvonalszervet, a ragadozók érzékelésen (Blaxter & Fuiman 1990; Fuiman 1993) kívül ismeretes, hogy ragadozók jelenlétében a rajképzés fontos viselkedés (Magurran 1990a) és a neuromasztok száma pozitívan függ össze a rajképzéssel (Pitcher et al. 1976; Partridge & Pitcher 1980).

4.1.4.3 Színlátás

A látópigment spektrális abszorbanciája leírja, hogy az adott pigment milyen hatásfokkal nyeli el a különböző energiájú fotonokat (azaz a különböző hullámhosszú fényt). Az összes látópigmentet egy G-protein-kapcsolt receptor és egy fehérjéhez (opszin) kötött fényelnyelő kromofór alkotja. A spektrális abszorbancia az opszin és a kromofór interakcióján múlik (Bridges 1972; Yokoyama & Yokoyama 2000). Az eltérő fényviszonyokhoz való alkalmazkodás történhet az opszin megváltoztatásán keresztül, amely mindig evolúciós folyamat, és ezért általában relatíve lassú (Terai et al. 2006; Jokela-Määttä et al. 2007, 2009; Larmuseau et al. 2009, 2010). Ennek alternatívája a kromofór-alapú alkalmazkodás, amely két kromofór változat (A1: 11-*cis* retinal; A2: 11-*cis* 3,4 dehydroretinal) arányának megváltoztatását jelenti, ami előfordulhat halaknál, kétéltűeknél és egyes hüllőknél (Bridges 1972). A kromofór-alapú hangolás képessége genetikailag meghatározott,

de amennyiben jelen van, akkor az egyedfejlődés alatt történik, azaz a fenotipikus plaszticitás egy formájának tekinthető (Wald 1946; Reuter et al. 1971; Temple et al. 2006).

A balti és Fennoskandináv régiók egy természetes kísérletnek tekinthetőek az adaptív divergencia tekintetében, mivel a Pleisztocén jégtakaró csak körülbelül 9000 éve kezdett visszahúzódni, lehetővé téve az addig refúgiumokba húzódott fajoknak, hogy a megnyíló változatos élőhelyeket benépesítsék (Donner 1995; Eronen et al. 2001). Igaz ez a vízi élőlények látására is. Mi a kilenctüskés pikók látópigmentjeinek spektrális változatosságára, és a változatosság mögött álló mechanizmusokra voltunk kíváncsiak.



19. ábra. Az A1:A2 kromofór arány kilenctüskés pikónál (átlag ± szórás). A) Édesvízi populációk. B) Tengeri populációk és MAS, egy kis tó ami csak pár évtizede izolálódott a Fehér-tengertől. A RYT populáció a két ismétlésben eltérő mintázatot mutatott. A * jel azokat a populációkat jelöli, ahol szignifikánsan eltért a csapok és pálcikák A1 kromofór aránya. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

Három tengeri, egy nagy tavi és négy izolált kis tavi kilenctüskés pikó populációt vizsgáltunk (Saarinen et al. 2012). Egy tengeri, egy nagy tavi és két

izolált kis tavi élőhelyen a környezeti fényviszonyokat is felmértük. A csapokban és pálcikákban mértük a fényelnyelési spektrumot. Meghatároztuk az A1:A2 kromofór arányt a csapokban és pálcikákban, valamint a pálcikák opszin génjének a bázissorrendjét.

Minden populációból három egyed (N = 24) opszin génjét szekvenáltuk, de csak két szinonim mutációt találtunk, tehát az opszin fehérje minden egyedben azonos lehetett. Ebből viszont az következik, hogy a megfigyelt fényelnyelési spektrum különbségek (2. táblázat) a pálcikákban kizárólag a kromofór-alapú hangolásból erednek. Négy populációban (három izolált kis tó és a nagy tó) a pálcikák és csapok A1 kromofór dominanciáját, a másik négy populációban (három tengeri és egy izolált kis tó) pedig A2 dominanciáját mutattuk ki (2. táblázat). Az A1 domináns csoportban a csapokban szignifikánsan nagyobb arányban voltak A2 kromofórok jelen mint pálcikákban, az A2 domináns populációknál nem volt ilyen elkülönülés (19. ábra).

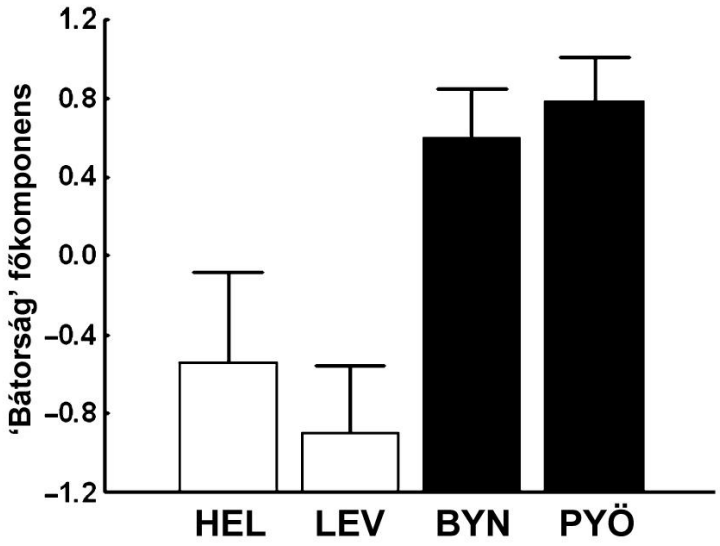
A leírt A1:A2 kromatofór mintázat ránézésre nem támogatja az általunk várt habitat-specificitást. Azonban az A2 kromofór dominanciájú izolált kis tó (Mashinnoje) közvetlen a Fehér-tenger partján található, a tengertől való izolációja mindössze évtizedekkel ezelőtt történt (Ziuganov & Zotin 1995; *White Sea Biological Station, Kartesh* személyes közlés), amit a fehér-tengeri mintákkal való genetikai egyezése is bizonyít (Shikano et al. 2010c). Tehát elképzelhető, hogy az itteni látásviszonyok még nem igényelték a kromofór arányok eltolását, amit a másik négy édesvízi (tengertől távoli, régen elvált) populációnál figyeltünk meg. De még ha el is fogadjuk a habitat-függő populációs szétválást, az eredményeink szöges ellentétben vannak az általánosan ismert trendekkel, azaz a vörös szín irányába eltolt érzékenységgel édesvízi tavakban (A2 dominancia) ami megfelel a környezeti körülményeknek (Bridges 1972; Jerlov 1976; Jokela-Määttä et al. 2007). Ezek az eredmények meglehetősen zavarba ejtőek, és a helyzet tisztázásához további vizsgálatok szükségesek több bevont populációval és *common garden* alapon. Érdekes viszont az A1 domináns populációknál megfigyelt A1:A2 arány eltérés a csapok és pálcikák között, ami szelektív kromofór feldolgozást jelez a különböző receptor típusokban. Ezt mi figyeltük meg elsőként. A jelenség habitat-függése biztató a jövő vizsgálatok szempontjából, hiszen valószínűvé teszi a szelektív kromofór feldolgozás adaptivitását.

Populáció; mintavétel éve; mintaszám (egyedek)	Fotoreceptor típusa; mintaszám (sejtek)	Abszorbancia maximum (nm) ± Standard hiba	A1-A2 kromofór arány (A1 típus százaléka)
ABB; 2008; 6	Pálcika; 107	507,8 ±0,6	98
	Csap; 126	519,2 ± 0,7	71
POR; 2008; 5	Pálcika; 137	511,0 ± 1,4	85
	Csap; 75	523,3 ± 1,7	60
PYÖ; 2008; 6	Pálcika; 108	504,8 ± 0,4	92
	Csap; 59	518,6 ± 0,6	68
PYÖ; 2011; 6	Pálcika; 225	507,9 ± 0,6	96
	Csap; 131	534,3 ± 1,8	40
RYT; 2008; 6	Pálcika; 180	507,9 ± 1,5	90
	Csap; 108	520,3 ± 1,0	73
RYT; 2011; 6	Pálcika; 148	516,5 ± 3,2	63
	Csap; 212	540,6 ± 1,6	36
BÖL; 2008; 6	Pálcika; 152	527,6 ± 1,3	20
	Csap; 92	547,7 ± 1,6	4
HEL; 2008; 5	Pálcika; 98	527,3 ± 1,4	5
	Csap; 78	538,0 ± 2,7	35
HEL; 2011; 6	Pálcika; 251	529,0 ± 0,6	11
	Csap; 242	544,8 ± 2,3	23
LEV; 2008; 3	Pálcika; 112	528,6 ± 1,4	51
	Csap; 31	536,0 ± 1,1	12
MAS; 2008; 6	Pálcika; 145	529,5 ± 0,6	7
	Csap; 95	543,5 ± 1,0	3

2. táblázat. Abszorbancia maximum és az A1:A2 kromofór arány nyolc kilenctüskés pikó populációban, a csapokban és pálcikákban. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

4.1.5 Viselkedés

Noha a viselkedés tekinthető a legplasztikusabb tulajdonságnak (West-Eberhard 2003), a geográfiai változatossága régóta ismert (Foster & Endler 1999; Foster 1999) és a heritabilitását is gyakran bizonyították (van Oers et al. 2005), tehát ugyanúgy evolválódhat, mint a kevésbé plasztikus tulajdonságok. Ráadásul, dacára a nagyfokú plaszticitásnak, napjainkra már egyértelművé vált, hogy a funkcionálisan különböző viselkedések (pl. kockázatvállalás, agresszió, aktivitás) sem függetlenek egymástól, hanem korrelációt mutathatnak az egyedeken keresztül. A jelenséget az emberi személyiség egyes vetületeihez való hasonlósága miatt viselkedési szindrómának neveztek el (Sih et al. 2004a,b). Ezek a szindrómák általában gyenge korrelációt jelentenek, de általánosan megfigyelhetők (Garamszegi et al. 2012). Amennyiben a különböző viselkedéseket szindrómaként kezeljük, szükség van a komplex viselkedési típus fogalmának (Bell 2007) és az azt reprezentáló, az egyedeket az összes vizsgált viselkedés szempontjából egyszerre leíró változó(k)nak a bevezetésére.



20. ábra. Populációs szétválás viselkedésben (átlag + 95% konfidencia intervallum) tengeri (üres oszlopok) és izolált kis tavi (kitöltött oszlopok) kilenctüskés pikóknál. A 'bátorság' főkomponens pozitívan korrelált az összes vizsgált viselkedési változóval (táplálkozási aktivitás, kockázatvállalás táplálkozási kontextusban, agresszió és kockázatvállalás explorációs kontextusban). A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

Nagyszámú viselkedési tulajdonságnál bizonyított a predációs nyomás nagyságának a viselkedést befolyásoló hatása (Magurran & Seghers 1991, 1994). Mivel a predáció komplex hatást fejt ki az életmenet *trade-off*-okra (Blanckenhorn 2000), feltehetően több viselkedést befolyásol egyszerre. Feltételeztük, hogy a predációs nyomás szempontjából extrém eltérést mutató kilenctüskés pikó populációk között a viselkedés is markánsan eltér. Az egyedfejlődés alatt szerzett tapasztalatok közvetlen hatásaira kontrollálandó, két tengeri és két izolált kis tavi populációból neveltünk halakat *common garden* körülmények között, ragadozókkal vagy fajtársakkal való interakciók, illetve ökológiai kényszerek nélkül. Mikor a halak

elérték az ivarérett méretet, négy viselkedést mértünk: táplálkozási aktivitást, kockázatvállalást táplálkozási kontextusban, agressziót és kockázatvállalást explorációs kontextusban (Herczeg et al. 2009b).

Főkomponens analízissel kreáltunk egy új változót, amely az összes eredeti változóval azonos irányba függött össze. Ez a változó leírta a viselkedési típust, mégpedig úgy, hogy az egyedeket egy közös bátorság-tengelyen (*shyness-boldness continuum*) helyezte el. Ennek a változónak az analízise egyértelmű eredményt adott; az egymástól geográfiailag és genetikailag is független populációk nem tértek el habitaton belül, a habitatok viszont egyértelműen különböztek (20. ábra). Az izolált kis tavakból származó pikók „bátrabbak" voltak, azaz gyorsabban kezdtek táplálkozni, nagyobb kockázatot vállaltak és agresszívebbek voltak, mint a tengeri fajtársaik (Herczeg et al. 2009b).

Mint az előző fejezetekben többször, itt is elmondható, hogy a közvetlen környezeti hatásoktól mentes habitat-függő populációs szétválás a természetes szelekció által irányított adaptív evolúciót jelzi Clarke 1975; Endler 1986; Schluter & Nagel 1995; McGuigan et al. 2005). Érdekes módon a ragadozók jelenléte-hiánya akár ellentétes hatással is lehet a préda viselkedésére. A gyakoribb – és intuitíve logikusabb – összefüggés az, amikor a magas predációs nyomást elszenvedő populációkban az átlagos viselkedési típus a kockázatkerülő irányba tolódik el a ragadozókkal való szembekerülés esélyét csökkentendő (Bell 2005; Brydges et al. 2008). Előfordul azonban az ellenkezője is. Brown et al. (2005, 2007) a püspökhal (*Brachyraphis episcopi*) vizsgálatánál azt találta, hogy a magas predációs nyomás alatt lévő populációkban a halak kockázatvállalóbbak voltak, és ez a különbség megmaradt a laboratóriumban nevelt generációban is. A szerzők interpretációja szerint magas predációs nyomás alatt nagyobb kockázatvállalásra van szükség, mint az alacsony predációval jellemezhető élőhelyeken a napi minimálisan szükséges aktivitás fenntartásához. A mi esetünk a gyakoribb szcenáriót támogatja. Ezek az eredmények tökéletesen illeszkednek az eredeti hipotézisünkhöz: a ragadozó és versenytárs halfajok nélkül evolválódó izolált kis tavi kilenctüskés pikók esetében az intraspecifikus kompetíció a rátermettséget leginkább befolyásoló környezeti faktor. A kompetíciós sikerhez pedig a nagy testméreten és a hosszú, de intenzív növekedési szakaszon át vezet az út. Mindezekhez viszont maximalizálni kell az energia-bevitelt, amihez aktívabb, agresszívebb és kockázatvállalóbb viselkedésre van szükség.

4.1.6 Fejlődési stabilitás

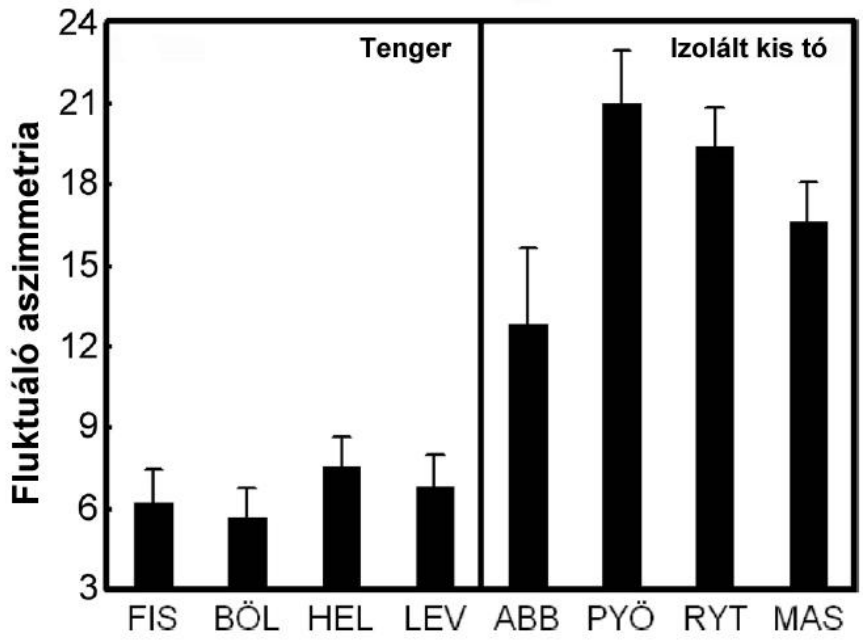
A bilaterális képletekben megfigyelhető aszimmetriát három fő csoportba oszthatjuk (Van Valen 1962; Palmer & Strobeck 1986): direkcionális aszimmetria, antiszimmetria és fluktuáló aszimmetria. A direkcionális aszimmetria egy adott oldal konzisztens megnagyobbodását jelenti, az antiszimmetria pedig egy random oldal megnagyobbodását. Az aszimmetria e két esete általában adaptív evolúció eredménye, azaz az aszimmetria előnyös. Ezzel szemben a fluktuáló aszimmetrián (FA) az egyedfejlődés alatti zavaró tényezők hatására létrejött, az optimális

szimmetriától való random eltéréseket értjük és ezért a fejlődési (in)stabilitás mérőszámaként is tekinthetjük (Van Dongen 2006). Ebből kiindulva, gyakran az adott egyedet ért stressz, vagy tágabb értelemben a rátermettség indikátora is lehet (Leary & Allendorf 1989; Parsons 1992; Clarke 1995; Leung & Forbes 1996; Leung et al. 2000; Van Dongen 2006). Sok vizsgálat eredménye támogatta a magas FA – alacsony rátermettség (pl. Martín & López 2000, 2001; Polak & Taylor 2007), illetve magas FA – magas stressz összefüggéseket (pl. Graham & Felley 1985; Sarre 1996; Siikamäki & Lammi 1998). Érdemes kiemelni, hogy a környezeti stressz mellett bizonyított a genetikai stressz (beltenyésztes, a genetikai változatosság csökkenése) és a FA pozitív kapcsolata is (Roldan et al. 1998). Ugyanekkor sok vizsgálat talált ellentmondó eredményeket, illetve a várt összefüggések hiányát. Ennek következtében a FA használhatóságáról a fejlődési (in)stabilitás leírására komoly viták zajlanak (Lens et al. 2001, 2002; Van Dongen 2006). Az ellentmondó eredmények és az FA ezt követő „népszerűség"-csökkenése legalább részben biztosan a módszertani problémáknak (Merilä & Björklund 1995; Palmer 1999) köszönhető, és ezért a megfelelő mintavételi és statisztikai módszerek alkalmazásával becsült FA mindenképpen releváns információt hordozhat akár egyedi, akár populációs szinten (Van Dongen 2006).

Elképzelhető, hogy populációs szinten nem csak a stresszben megfigyelhető különbségek, hanem az aszimmetria ellen ható szelekciós erők eltérései is okozhatnak szétválást az FA mértékében. Ezt a hipotézist közvetlen módon még nem tesztelték, de az összefüggés miszerint a funkcionálisan fontos tulajdonságok fluktuáló aszimmetriája tipikusan alacsonyabb, mint a kevésbe fontos tulajdonságoké (Karvonen et al. 2003) mindenesetre támogatja. Az általunk vizsgált kilenctüskés pikó rendszer kiváló modell a hipotézis predikcióinak tesztelésére, és a tengeri *vs.* izolált kis tavi pikópopulációk aszimmetriájának összehasonlításával fontos biológiai információt nyerhetünk a rendszerről.

A mechanoszenzoros oldalvonalszerv (lásd 4.1.4.2) egy kiváló tulajdonságcsoport az aszimmetria vizsgálatához. Az oldalvonalakat alkotó neuromasztok könnyen számolhatók, és tudjuk, hogy a számolható tulajdonságok jobban használhatóak az FA vizsgálatában, mint a mérendő tulajdonságok (Lens et al. 2001), mivel minimális hibával becsülhetőek (Herczeg et al. 2005). Ráadásul az oldalvonalszerv számos különálló oldalvonalból áll, és az FA használhatósága jelentősen javul, ha nem egy, hanem több tulajdonság aszimmetriáján alapszik (Van Dongen 2006). Végezetül, az oldalvonalszerv egy fontos érzékszerv, aminek precizitása nagy valószínűséggel befolyásolja a rátermettséget; elég csak a táplálkozásban (Montgomery & MacDonald 1987; Montgomery & Milton 1993), a ragadozó-elkerülésben (Fuiman 1993) és a rajképzésben (Partridge & Pitcher 1980) betöltött szerepére gondolnunk. Jelen vizsgálatunkban négy tengeri és négy izolált kis tavi kilenctüskés pikó populáció fluktuáló aszimmetriáját (12 számolható bilaterális tulajdonság alapján: 11 oldalvonal + az oldalsó pajzsok) és genetikai

heterozigóciáját (23 neutrális mikroszatellita lókusz alapján) hasonlítottuk össze (Trokovic et al. 2012).



21. ábra. Összesített fluktuáló aszimmetria (12 bilaterális jelleg alapján) nyolc kilenctüskés pikó populációban (átlag ± standard hiba). A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

A tengeri populációk átlagos heterozigóciája kétszer nagyobb volt, mint a kis tavi populációké (tenger: H_E = 0,58; standard hiba = 0,06; kis tó: H_E = 0,30; standard hiba = 0,06). Ez az eredmény egybevág a nagyobb mintán végzett populációgenetikai analíziseinkkel (Shikano et al. 2010c). Az FA mértéke az izolált kis tavakban majdnem háromszorosan meghaladta a tengeri populációkban mértet (21. ábra). Ez a hatás a heterozigóciától, ivartól és a populációktól (habitaton belül) függetlenül jelentkezett.

Az izolált kis tavakban evolválódott, ragadozó elleni védelmi struktúráit redukáló vagy elvesztő, nagy növekedési erélyű, agresszív, hosszú életű pikóknál tehát rendkívül magas fluktuáló aszimmetriát találtunk, amit a kis tavakra jellemző alacsony genetikai változatosság egyedül nem magyarázott. A mintázat tehát – közvetve ugyan – a szelekciós hipotézisünket támogatta. A ragadozók általi szelekció a természetes szelekció egyik fő hajtóereje, és több vizsgálat is bizonyította, hogy a ragadozóknak áldozatul esett egyedek magasabb FA indexszel jellemezhetőek, mint a túlélők (Swaddle 1997; Bergstrom & Reimchen 2003; Galeotti et al. 2005). Sőt, az idősebb egyedek általában szimmetrikusabbak, mint a fiatalok (Moran et al. 1997). A ragadozók legalább három módon csökkenthetik a populációk átlagos FA szintjét. Például okozhatnak természetes szelekciót a szimmetrikusabb egyedek irányába. Ehhez azonban a fluktuáló aszimmetriának örökölhetőnek kellene lennie, amire nincs sok bizonyíték (Leamy 1997; Fuller & Houle 2003). Amennyiben az FA genetikai háttértől függetlenül az adott egyed növekedési környezetét reflektálja, a ragadozók

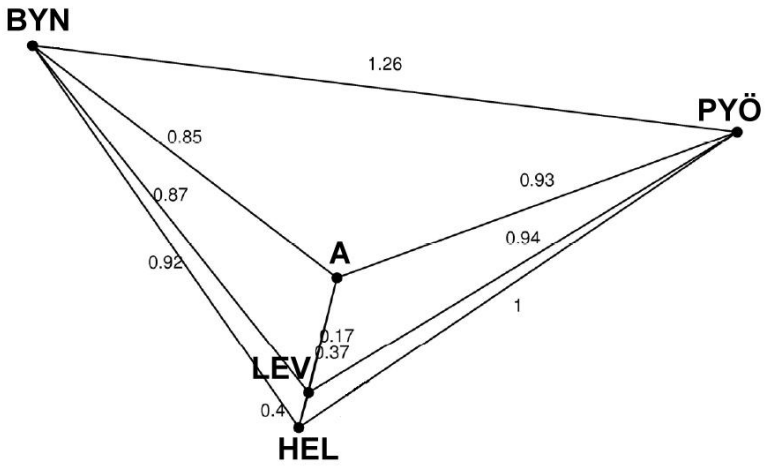
még mindig kiemelhetik a magas aszimmetriájú (és gyengébb minőségű) egyedeket. Végezetül, feltéve, hogy a predációs nyomás drasztikus csökkenése óriás méret és megnövekedett növekedési erély evolúciójához vezethet (Herczeg et al. 2009a; 2012; Aikio et al. 2013), az extrém növekedési sebesség pedig önmagában is okozhat megnövekedett fejlődési instabilitást (Mangel & Munch 2005), kirajzolódik előttünk egy harmadik, közvetett predáció – FA kapcsolat. Függetlenül a mechanizmustól, a predációs nyomás csökkenése az FA csökkenéséhez vezethet. Különösen igaz ez olyan tulajdonságokra, melyek fontosak a ragadozó elkerülésében, vagy a ragadozó támadásának túlélésében. Az oldalvonalszerv pedig egy éppen ilyen jelleg, szerepe a ragadozók elkerülésében (Blaxter & Fuiman 1990; Fuiman 1993) és a ragadozók elkerülésében fontos rajképzésben (Partridge & Pitcher 1980) egyaránt ismert. Az oldalsó pajzsok tekintetében az oldalvonalakkal megegyező mintázatot találtunk. Bár a kilenctüskés pikó oldalsó pajzsainak funkcionális szerepe kicsiny méretükből adódóan megkérdőjelezhető, a ragadozó halak nélküli izolált kis tavakban a pajzsok száma csökkent (Herczeg et al. 2010a). Összességében minden eredményünk támogatja a hipotézisünket miszerint a predációs nyomás csökkenése a szimmetriát fenntartó szelekció lazulásához, és ennek eredményeképpen a fluktuáló aszimmetria növekedésében tetten érhető megnövekedett fejlődési instabilitáshoz vezet. Egy nem kizáró alternatív magyarázat szerint az izolált kis tavakban a ragadozók és interspecifikus kompetítorok hiányában az óriás méret és a maximalizált növekedési sebesség olyan fontos, hogy az így nyert előny meghaladja a fejlődési instabilitásban jelentkező költséget. A jelenség teljesebb megértéséhez azonban további kísérletes munkára lesz szükség.

4.1.7 A természetes szelekció szerepének tesztelése

A természetben megfigyelt habitat-függő populációs különbségek evolúciós interpretációjával legalább két szinten lehet probléma. Először el kell választani a genetikailag meghatározott eltéréseket a fenotipikus plaszticitástól (Conover & Schultz 1995; Merilä 2009). Ezt például *common garden* kísérletekkel lehet megtenni. Optimális esetben több generációt és komplex keresztezési sémákat kellene alkalmazni a legjobb felbontáshoz, de a legtöbb fajnál egyszerű keresztezésekre és egy generációra támaszkodunk. Amikor a szétválás mögötti genetikai komponens bizonyított, jön a következő kérdés: a szétválás a természetes szelekciónak vagy véletlennek (random genetikai sodródás) a következménye (Merilä & Crnokrak 2001; Leinonen et al. 2008)? Itt a populációs különbségek véletlentől való eltérése, például a vizsgált tulajdonság valamilyen környezeti változóval való korrelációja adhat támpontot.

A természetes szelekció és a random sodródás populációs szétválásban viselt szerepeinek bizonyító erejű tesztelésére is van mód. A nem-modell fajok esetében talán legelterjedtebb módszer a neutrális genetikai szétválás (F_{ST}) és a kvantitatív genetikai szétválás (Q_{ST}) összehasonlításán alapul (Spitze 1993; Merilä 1997). Az F_{ST}-t tekinthetjük a random genetikai folyamatok által előidézhető szétválás mércéjének. A $Q_{ST} – F_{ST}$ összehasonlításoknak három potenciális kimenetele lehet

(Merilä & Crnokrak 2001; Leinonen et al. 2008): (i) $Q_{ST} > F_{ST}$, azaz a vizsgált tulajdonságban megfigyelt divergencia meghaladja a neutrális szintet és ez bizonyítja a szétválasztó szelekció szerepét, (ii) $Q_{ST} = F_{ST}$, azaz a vizsgált tulajdonságban megfigyelt divergencia nem különbözik a neutrális szinttől és így nem tudjuk bizonyítani a természetes szelekció szerepét és (iii) $Q_{ST} < F_{ST}$, azaz a vizsgált tulajdonságban megfigyelt divergencia alacsonyabb a neutrális szintnél, ami bizonyítja a stabilizáló szelekciót. A módszer kritikus része, hogy a fenotípusos divergencia genetikai komponensével becsüljük a Q_{ST}-t, amihez *common garden* kísérletre vagy ismert pedigrékre van szükség (Lynch & Walsh 1998). Ráadásul az eredeti módszer csak nagyszámú populáció bevonásával működik megfelelően (O'Hara & Merilä 2005). Időközben lehetőség nyílt hierarchikus megközelítésre is (habitat-struktúra figyelembevétele; Chapuis et al. 2008). A nagyszámú populáció szükségessége, különösen a *common garden* adatok vagy pedigrék igénye miatt továbbra is határt szabott a $Q_{ST} – F_{ST}$ összehasonlítások széleskörű alkalmazásának.



22. ábra. Neutrális genetikai távolságok a vizsgált populációk, és az adatokból becsült hipotetikus közös ős (A) között. A távolságok egysége egy hipotetikus ősi neutrális jelleg varianciája, így az ábrán látható viszonyok a megfigyelt fenotípusos szétválás null modelljének is tekinthetőek, azaz ilyen mértékű fenotípusos szétválást várnánk random genetikai sodródás következményeként, természetes szelekció nélkül. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

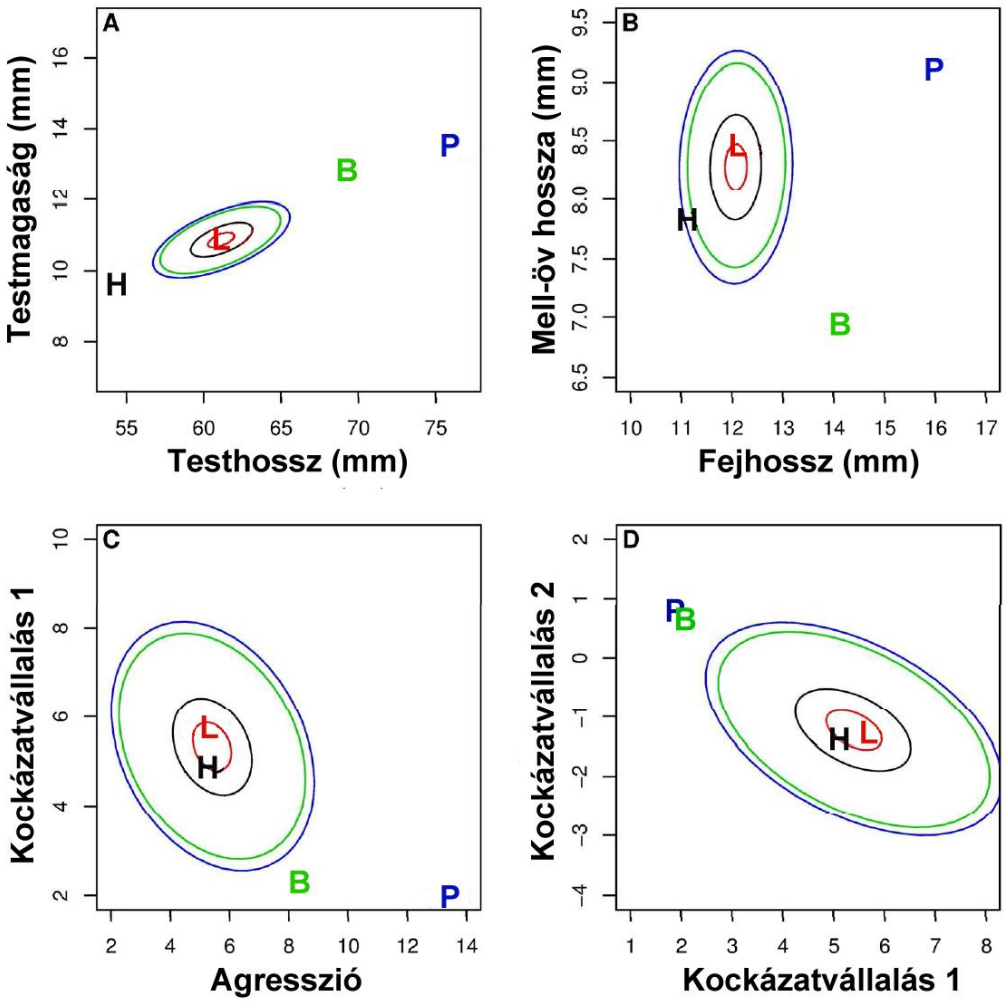
Az itt bemutatott vizsgálatunk fő célja egy alacsony mintaszámmal is jól működő és környezeti változókat is figyelembe vevő statisztikai módszer kidolgozása volt, ami magas F_{ST}-nél (azaz magas neutrális szétválásnál) is nagy statisztikai erővel bír. A módszert négy kilenctüskés pikó populáció (két tengeri és két izolált kis tavi) *common garden* adatain teszteltünk (Karhunen et al. 2014). A disszertáció szempontjából az aktuális eredmények a fontosak, hiszen a módszer tesztelésével életmenet, morfológiai és viselkedési tulajdonságoknál vizsgáltuk, hogy bizonyítható-e a szétválasztó szelekció szerepe?

A neutrális genetikai divergencia magas volt (F_{ST} = 0,35, 95% konfidencia-intervallum = 0,31-0,38). A két tengeri populáció (Balti- és Fehér-tenger) egymáshoz és a hipotetikus közös őshöz genetikailag közel állt, a két izolált kis tavi populáció (több mint 500 km-es távolsággal elválasztva) pedig mind egymástól, mind a tengeri populációktól és a közös őstől eltávolodott (22. ábra). A szétválasztó szelekció testméretben, testalakban, csontos védelmi struktúrákban és viselkedésben is tetten érhető volt. Érdekes, hogy amíg az életmenet és morfológiai változók tekintetében a fehér-tengeri populáció reprezentálta az ősi formát, és a balti-tengeri és kis tavi populációk – ha ellentétes irányba is változtak – egyaránt leszármazottnak tűnnek, a viselkedés tekintetében mindkét tengeri populáció igen hasonló és az ősinek tekinthető fenotípust mutatott, amitől a kis tavi populációk hasonló irányba mozdultak el (23. ábra).

Az új statisztikai megközelítés (DRIFTSEL, R kód szabadon hozzáférhető: http://www.helsinki.fi/biosci/egru/software/driftsel.html) alacsony számú populáció és magas F_{ST} mellett is nagy statisztikai erővel bíró módszernek bizonyult. Az eddig tárgyalt vizsgálatokban sikerült kimutatnunk habitat-specifikus populációs szétválást egy sor rátermettséget befolyásoló tulajdonságban (pl. testméret, viselkedés, morfológia), *common garden* adatokkal is támogatva. A feltehetően genetikailag meghatározott habitat-függő populációs szétválást tekinthetjük a természetes szelekció irányította adaptív evolúció jelének (Clarke 1975; Endler 1986; Schluter & Nagel 1995; McGuigan et al. 2005). Az ilyen alapon levont következtetések ugyan nagy valószínűséggel helyesek, de szigorúan véve nem bizonyító erejűek. Az új megközelítéssel viszont sikerült a szétválasztó természetes szelekció szerepét bizonyítanunk a testméretnél, testalaknál, védelmi struktúráknál és viselkedésnél, és így megerősítenünk az eddigi evolúciós interpretációnkat a többször, egymástól függetlenül kialakult izolált kis tavi kilenctüskés pikó fenotípusról.

4.1.8 Összegzés

Az első, populációs összehasonlításokon alapuló fejezetben bemutattam, hogy a ragadozó és kompetítor halfajoktól mentes izolált kis tavi környezetben több független esetben is hasonló fenotípusú kilenctüskés pikók alakultak ki. A kis tavi fenotípus szinte tökéletesen megfelelt a várakozásainknak: redukált ragadozó ellenes morfológiai struktúrák, óriás testméret, az ennek elérését segítő növekedési stratégia, késleltetett ivarérés, „bátor” viselkedési típus, minimális energiaraktárak, gyengébben fejlett központi idegrendszer, redukált oldalvonalszerv, megnövekedett fejlődési instabilitás és az óriás testméretből eredő többszörösére nőtt fekunditás jellemezte. A legtöbb, természetes populációkban megfigyelt mintázatot *common garden* körülmények között is reprodukálni tudtuk, ami a populációs szétválás genetikai meghatározottságát jelzi. Ez, a mintázat habitat-függő mivoltával együtt a természetes szelekció szerepére utal, amit a legfontosabb morfológiai/életmenet/viselkedési tulajdonságok esetében egy formális Q_{ST} – F_{ST} összehasonlítás is megerősített.



23. ábra. Fenotípusos szétválás kvantitatív tulajdonságokban, A, B) morfológiai jellegek, C, D) viselkedési tulajdonságok. HEL = H (fekete), LEV = L (piros), BYN = B (zöld), PYÖ = P (kék). A betűk a populációk additív genetikai (fenotípusos) átlagát jelölik, az ellipszisek pedig a medián differenciálódás határait random genetikai sodródás mellett. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

A mintázat mögötti ultimális evolúciós okként az izolált kis tavakban a ragadozó és kompetitor halfajok hiánya miatt döntővé vált intraspecifikus kompetícióban elért maximális sikert célzó szelekciós erőket sejtem. Ez az elképzelés kísérletesen nem bizonyított, bár véleményem szerint messze a legvalószínűbb a lehetőségek között, és a növekedési stratégia esetén a matematikai modellünk is támogatta. Mindenesetre egy sor rátermettséget nagy valószínűséggel befolyásoló kvantitatív tulajdonságnál találtunk szisztematikus, habitat-függő populációs szétválást, ami a vizsgált rendszert egy ígéretes evolúciós modellé emeli, és további vizsgálatokat tesz szükségessé.

Fontosnak tartom, hogy fajon belüli populációs összehasonlításokat sikerült végeznünk olyan tulajdonságok tekintetében is, ahol ez ritkaságszámba ment (agy és az érzékszervek). Ennek a jelentősége talán az agy méretének és struktúrájának

evolúciós vizsgálatában a legkézzelfoghatóbb, az elmúlt években egyre több ilyen típusú vizsgálaton alapuló publikáció jelent meg. A megközelítésben rejlő lehetőségeket és az eddigi eredményeket egy összefoglaló cikkben tettük közzé (Gonda et al. 2013).

4.2 Fenotípusos plaszticitás

A második fejezetben az adaptív szétválásban releváns környezeti tényezők manipulálására adott egyedfejlődés alatti választ, azaz a fenotipikus plaszticitást fogom tárgyalni. Pigliucci (2005) szerint a fenotipikus plaszticitás evolúciójának megértéséhez kulcsfontosságúak a különböző környezeti tényezőkhöz alkalmazkodott populációkban megfigyelhető plaszticitás mértékének összehasonlítását célzó vizsgálatok. Ennek megfelelően, az ilyen összehasonlító munkák száma az elmúlt években növekedésnek indult (pl. Wund et al. 2008; Edgell et al. 2009; Crispo & Chapman 2010a,b; Harris et al. 2011; Lind et al. 2011; Torres-Dowdall et al. 2012). Azok a populációs összehasonlítások, ahol a fenotipikus plaszticitás indukálásáért manipulált környezeti változók és a populációk közötti adaptív evolúciós szétválásért felelős környezeti változók azonosak, még mindig meglehetősen ritkák (Van Buskirk & Arioli 2005; Rogell et al. 2012; Torres-Dowdall et al. 2012).

Figyelembe véve a (közvetve) bizonyított kiindulási feltételezésünket, miszerint az izolált kis tavakban a predáció és interspecifikus kompetíció drasztikus csökkenését követve az intraspecifikus kompetícióban elért siker vált a rátermettség növelésének kulcsává, a ragadozók és a fajtársak jelenlétét, és az elérhető táplálék mennyiséget manipuláltuk *common garden* körülmények között tengeri és kis tavi pikóknál. Kíváncsiak voltunk magára a fenotipikus plaszticitásra és a fenotipikus plaszticitás populációs különbségeire is. Ahol lehetett, vizsgáltuk az ivarok közötti eltéréseket is.

4.2.1 Ragadozó jelenlét/hiány és a táplálék mennyisége

A predáció nagy hatással bír a prédapopuláció életmenetére és dinamikájára (Roff 1992). Nagyszámú ragadozók elleni védelmet szolgáló tulajdonság evolúciója ismert (Tollrian & Harvell 1999). A predációs nyomás hirtelen (nem evolúciós léptékű) változásakor az evolúciósan fixált tulajdonságok komoly költségként jelentkezhetnek (Harvell 1990; Baker et al. 2010). Ezeket a költségeket csökkentheti vagy akár meg is szüntetheti a fenotipikus plaszticitás (West-Eberhard 2003). Például új élőhelyek kolonizációjakor változhat drasztikusan a predációs nyomás. A kis tavak izolációjakor tipikus jelenség a predáció csökkenése. A ragadozók által fenntartott szelekciós nyomás eltűnésekor a ragadozóellenes tulajdonságok evolúciósan redukálódhatnak (Blumstein & Daniel 2003; Messler et al. 2007; Lahti et al. 2009). Bonyolultabb a helyzet a ragadozóellenes tulajdonságok plaszticitásával. Ragadozó hiányában nem indukálódik plasztikus válasz, és így a ragadozóellenes fenotípus nem lehet közvetlen szelekció alatt. Ilyenkor a ragadozóellenes fenotípus ellen csak

közvetett szelekció léphet fel, a plaszticitás képességének költségein keresztül (Masel et al. 2007; Hall & Colegrave 2008; Lahti et al. 2009).

A ragadozók által kiváltott fenotipikus plaszticitást leginkább a morfológiai tulajdonságok köréből ismerjük rendkívül sok taxonnál (Brönmark & Miner 1992; Tollrian 1995; Van Buskirk & Schmidt 2000; Teplitsky et al. 2005; Hammil et al. 2008). A morfológiai tulajdonságok mellett szép számmal akadnak példák az életmenet vagy viselkedési tulajdonságok köréből is, a ragadozók jelenlétében a zsákmányfaj egyedei általában csökkentik az aktivitásukat, aminek köszönhetően csökken az energiafelvételük és ez negatívan befolyásolva a növekedésüket és szaporodási sikerüket (Werner et al. 1993; Biro et al. 2004; 2006). A nehezebben vizsgálható tulajdonságok köréből (pl. agystruktúra, érzékszervek) már jóval kevesebb ismeret áll a rendelkezésünkre.

Az egyedfejlődés alatt rendelkezésre álló táplálék mennyiségének hatása a viselkedésre és az életmenet-komponensekre közismert (Wooton 1973; Stearns 1992; Arendt & Reznick 2005; Ward et al. 2006; Dmitriew 2011). Ráadásul az elérhető táplálékmennyiség gyakran befolyásolja a ragadozókra adott plasztikus választ (Bolnick & Preisser 2005). A ragadozók elkerülését szolgáló aktivitás-csökkentés csak kis mértékben figyelhető meg amennyiben kevés a táplálék, ilyenkor a prédaállatok vállalják a kockázatot a növekedésük fenntartása érdekében (Biro et al. 2004, 2005). A ragadozók jelenlétére adott morfológiai válasz erősségét pozitívan befolyásolhatja a táplálék mennyisége (Teplitsky et al. 2005; Chivers et al. 2007; Pauwels et al. 2010).

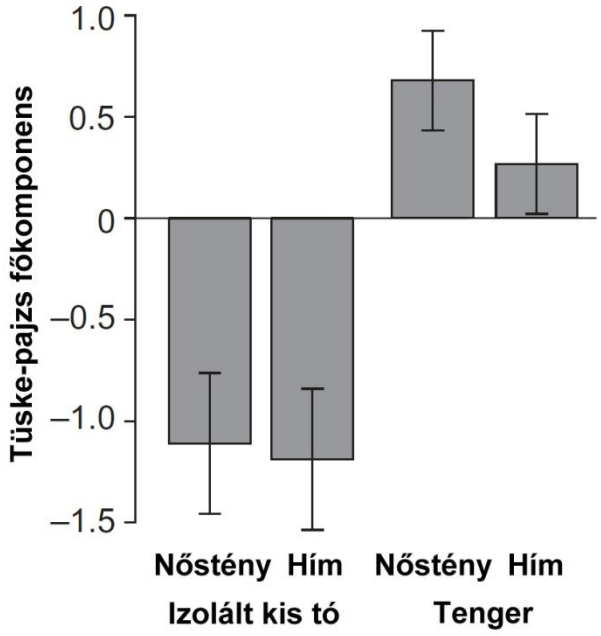
A kilenctüskés pikóknál megfigyelt predáció- és kompetíció-alapú habitat-függő evolúciós szétválás számos rátermettséget befolyásoló tulajdonságban kiváló modellé teszi a rendszert a ragadozó/táplálék manipuláció kiváltotta fenotipikus plaszticitás populációs összehasonlítására. Az alább tárgyalt vizsgálatokban négy tengeri és két izolált kis tavi populáció egyedeit szaporítottuk *in vitro*, és az F1 generációt neveltük egy faktoriális elrendezésű *common garden* kísérletben két predációs nyomás × két táplálékmennyiség kezelés mellett.

Általánosságban feltételeztük, hogy a változatos összetételű és feltételezhetően változatos predációs nyomást produkáló ragadozóhal-közösségekhez adaptálódott tengeri kilenctüskés pikó populációkban fogunk nagyobb ragadozó-indukálta plaszticitást találni, mivel az adott környezeti faktor tér- és időbeli változatossága hivatott a fenotipikus plaszticitást fenntartani (Moran 1992). Ugyanakkor a ragadozó ellenes tulajdonságok alacsony kifejeződése mellett minimális ragadozó indukálta plaszticitást is vártunk a ragadozó halak hiányához alkalmazkodott izolált kis tavi populációkban, mivel a változatosság (és egyben a kiváltó inger) hiánya a költséges plaszticitás csökkenéséhez vezethet például a genetikai asszimiláció mechanizmusán keresztül (Crispo 2007; Pfennig et al. 2010). A kis tavi, intraspecifikus kompetícióhoz alkalmazkodott populációkban a felvehető táplálék mennyiségének erős hatását vártuk az előbb említett okokból (Moran 1992). Ezen kívül feltételeztük a

ragadozók kiváltotta plaszticitás erősségének pozitív összefüggését is az elérhető táplálék mennyiségével. A vizsgálatban egy sor releváns tulajdonságot vontunk be, melyeknél intuitíve eltérő plaszticitást vártunk a következő sorrendben: morfológia < idegrendszer ≈ életmenet < viselkedés.

4.2.1.1 Morfológia

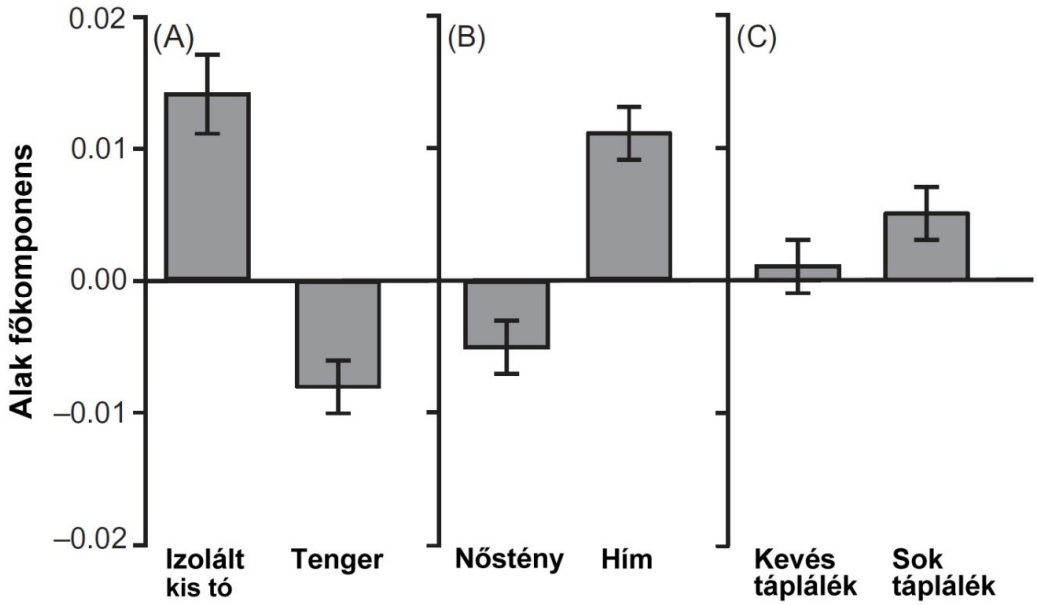
A predáció számos fixált vagy plasztikus ragadozóellenes morfológiai karakter evolúciójához vezetett (Tollrian & Harvell 1999), ilyenek például a pikók tüskéi és pajzsai (Reimchen 1983; Bell & Foster 1994; Leinonen et al. 2011). A limnetikus – bentikus habitatváltás befolyásolja a pikók testalakját; limnetikus környezetben az áramvonalas, míg bentikus környezetben inkább a mély, robosztus alak az előnyös (Walker 1997; Bergstrom 2002; Walker et al. 2005). Korábban (4.1.2; Herczeg et al. 2010a) bemutattam, hogy (i) az izolált kis tavakban, ahol nincsenek szimpatrikus ragadozó halak, a kilenctüskés pikók ragadozóellenes morfológiai képletei (tüskék és pajzsok) redukálódnak vagy akár el is tűnhetnek, illetve (ii) az izolált kis tavak bentikus környezetében a pikók testalakja robosztusabb, mint a tengerekben vagy nagy tavakban. Jelen vizsgálatunkban (Välimäki et al. 2012) a kilenctüskés pikók morfológiájának ragadozó-indukálta plaszticitására, eme plaszticitás táplálékellátottság-függésére, illetve a plaszticitás habitatok és nemek közötti eltérésére voltunk kíváncsiak.



24. ábra. Változatosság (korrigált átlag ± standard hiba) a kilenctüskés pikók csontos fegyverzetében (hasi tüske, mell-öv és pajzsok) egy tengeri és izolált kis tavi populációkon végzett, ragadozó jelenlét/hiányon és alacsony/magas táplálékellátottságon alapuló faktoriális *common garden* kísérlet alapján. Az itt ábrázolt főkomponens az erősen redukált fegyverzettől (alacsony értékek) a maximálisan kifejezett fegyverzetig (magas értékek) terjedő gradienst írja le. Szignifikáns habitat × ivar interakció.

A fő testalak tengelyek ugyanazok voltak, mint a korábbi vizsgálatainkban (Herczeg et al. 2010a; Turtiainen, Herczeg, Merilä kézirat), a biológiailag releváns változatosság legnagyobb részét a test mélységének és a faroknyél hosszának változása tette ki (lásd a 8. ábrán bemutatott mintázatokat). A ragadozó-kezelésnek semmilyen hatása nem volt a halak morfológiájára. A táplálék-kezelésnek is csak egy gyenge hatását figyeltük meg a test mélységére. A korábbi eredményeinket ebben a *common garden* kísérletben is sikerült reprodukálnunk: az izolált kis tavi pikók redukált fegyverzettel és mélyebb, robosztusabb testalakkal rendelkeztek, mint a tengeriek, és a hím pikók fegyverzete gyengébb, testalakja pedig mélyebb, robosztusabb volt, mint a nőstényeké (24. & 25. ábrák).

Az eredményeink egyértelműek voltak, a ragadozó kezelésünk nem befolyásolta a pikók morfológiáját, habitattól, ivartól és a táplálék-kezeléstők függetlenül (Välimäki et al. 2012). A plaszticitás hiányának egy sor potenciális oka lehet. A legelső lehetőség rögtön a kísérleti elrendezés hibája: biztosan érzékelték a szituáció potenciálisan veszélyes mivoltát a ragadozó kezelésbe tartozó pikók? Sok esetben a ragadozón kívül a megtámadott fajtárs kémiai vészjelzései is elengedhetetlenek a plasztikus válasz kiváltásához (Brönmark & Petterson 1994; Wisenden & Chivers 2006). A közeli rokon háromtüskés pikóknál azonban Frommen et al. (2011) bizonyította a csapósügér szaganyagaira adott morfológiai választ a fajtársak vészjelzései nélkül is. Mi is csapósügér szaganyagokat használtunk, kérdés persze, hogy egyáltalán érzékelték-e a sügéreket a kilenctüskés pikóink? Pusztán a morfológiai eredményeink alapján erre nem tudnánk választ adni, de a későbbiekben tárgyalt életmenet, idegrendszer vagy viselkedés esetében a pikók reakciója a sügérek szaganyagaira egyértelmű volt (Herczeg & Välimäki 2011; Gonda et al. 2012; Välimäki & Herczeg 2012), tehát nagy valószínűséggel a morfológiai plaszticitás hiánya nem műtermék. Elképzelhető lehetne, hogy a vizsgált morfológiai tulajdonságoknak a jelentősége minimális a ragadozók elleni védelemben, és ezért a plaszticitásuk költsége meghaladná az előnyöket és ez a plaszticitás hiányát indokolná (DeWitt et al. 1998). Ezt a magyarázatot is el kell vetnünk, hiszen a faj populációi között megfigyelt habitat-specifikus morfológiai változatosság (pl. Herczeg et al. 2010a; Mobley et al. 2011) és a kilenctüskés pikók védelmi struktúráinak kísérletesen bizonyított fontossága (Hoogland et al. 1957; Ziuganov & Zotin 1995) egyaránt bizonyítja a morfológiai változatok adaptivitását. Figyelembe véve a vizsgált morfológiai tulajdonságok heritabilitását pikóknál (Blouw & Boyd 1992; Leinonen et al. 2010), levonhatjuk a következtetést, miszerint a kilentüskés pikó morfológiája evolúciósan flexibilis mind populációk között (habitat-függő mintázatok), mind populációkon belül (ivari dimorfizmus), ám a genetikailag meghatározott fenotípus az egyedfejlődés alatt merev.



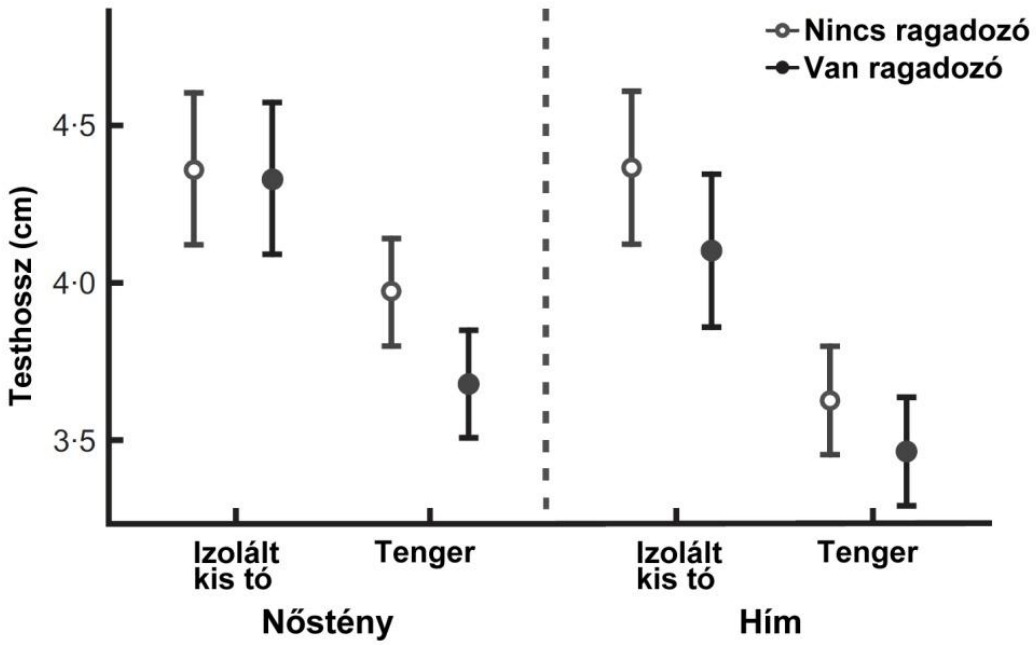
25. ábra. Változatosság (korrigált átlag ± standard hiba) a kilenctüskés pikók testalakjában egy tengeri és izolált kis tavi populációkon végzett, ragadozó jelenlét/hiányon és alacsony/magas táplálékellátottságon alapuló faktoriális *common garden* kísérlet alapján. Az iránypont-alapú geometriai morfometria analízis eredményeként kapott alak főkomponens egy gradienst ír le a keskeny, áramvonalas testű és hosszú faroknyelű pikóktól (alacsony értékek) a robosztus, mély testű és rövid faroknyelű pikókig (magas értékek). Szignifikáns hatások: A) habitat, B) ivar, C) táplálék-kezelés.

4.2.1.2 Életmenet

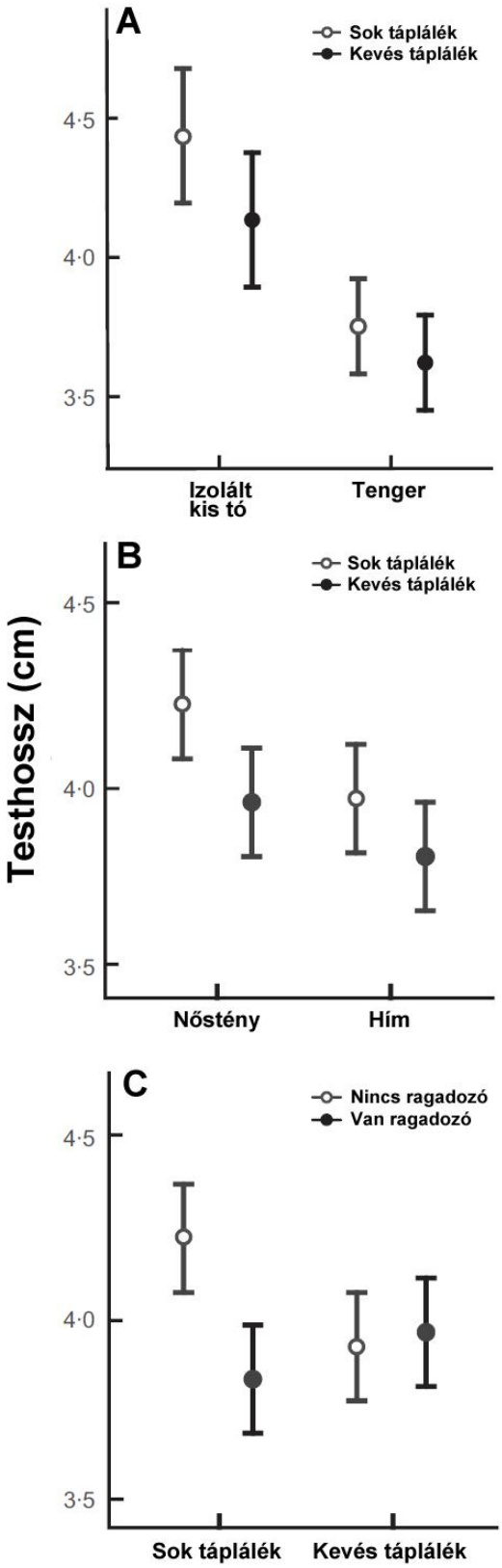
A fenotipikus plaszticitás vizsgálatába vont életmenet változóknál (testméret, növekedés, energiaraktárak) korábban egyértelmű habitat-függő populációs szétválást találtunk: az izolált kis tavi kilenctüskés pikók több független esetben is óriási mérettel, jelentősen meghosszabbodott növekedési időszakkal és még *ad libitum* elérhető táplálék mellett is minimális energiaraktárakkal rendelkeztek (4.1.3; Herczeg et al. 2009a; 2012; Välimäki, Herczeg, Merilä kézirat). Ezt a szimpatrikus ragadozó és kompetitor halfajok hiányával és az ebből eredő megnövekedett fontosságú intraspecifikus kompetícióval magyaráztuk; az ilyen környezetben evolválódó pikók a maximális testméret elérésére törekednek minden elérhető energia befektetésével, és akár az ivarérés idejének kitolásával is (Ab Ghani et al. 2013). Ezzel szemben a tengeri pikóknak a jelentős predációs nyomásnak köszönhetően a lehető legkorábbi szaporodás, akár a kis testméret árán, a fő faktor a rátermettségük növelésében (Aikio et al. 2013). Az életmenet tulajdonságok természetes változatosságának megértéséhez fontos lépcső a genetikailag meghatározott populációs mintázatok mellett a környezet által az egyedfejlődés alatt kiváltott változatosság feltárása is mind a populációk, mind a nemek között.

4.2.1.2.1 Testméret

A ragadozó kezelésünk hatása a testméretre habitat- és ivar-függő is volt (Välimäki & Herczeg 2012), a tengeri pikók és a kis tavi pikók hímjei egyaránt kisebbek lettek a csapósügér szaganyagait tartalmazó vízben növekedve, a kis tavi pikók nőstényeit viszont nem befolyásolta a kezelés (26. ábra). A táplálék kezelés hatása is habitat-specifikus volt, a kis tavi pikók hatékonyabban használták ki az *ad libitum* táplálékmennyiséget, mint a tengeri fajtársaik (27/a. ábra). A táplálék kezelésnél az ivar hatása független volt a habitattól: a nőstény pikók növekedését erősebben befolyásolta az elérhető táplálék mennyisége, mint a hímekét (27/b. ábra). A két kezelésünk között szignifikáns interakciót is detektáltunk, az *ad libitum* táplálékellátottság növekedésre kifejtett pozitív hatása csökkent a ragadozó szaganyagainak jelenlétekor (27/c. ábra).



26. ábra. Habitat, ivar és ragadozó kezelés hatása kilenctüskés pikók méretére (korrigált átlag ± standard hiba) egy tengeri és izolált kis tavi populációkon végzett, ragadozó jelenlét/hiányon és alacsony/magas táplálékellátottságon alapuló faktoriális *common garden* kísérlet alapján.



27. ábra. Kettős interakciók hatása kilenctüskés pikók méretére (korrigált átlag ± standard hiba) egy tengeri és izolált kis tavi populációkon végzett, ragadozó jelenlét/hiányon és alacsony/magas taplálékellátottságon alapuló faktoriális *common garden* kísérlet alapján. A) habitat × táplálék, B) ivar × táplálék, C) táplálék × ragadozó hatások.

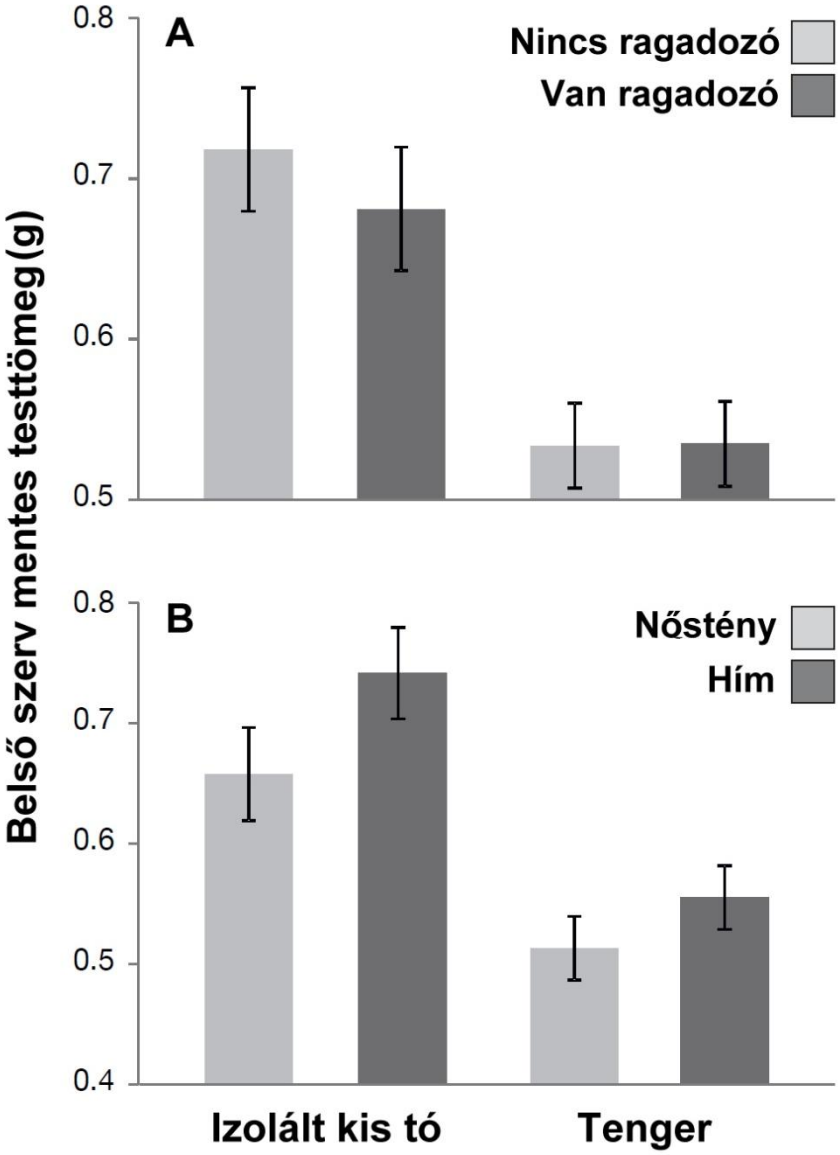
A ragadozók okozta kockázat általában a növekedési ráta, és végső soron a testméret csökkenését vonja maga után, mivel a zsákmányállat a ragadozókkal való találkozás esélyét csökkentendő minimalizálja az aktivitását és ezért kevesebb táplálékot tud felvenni (pl. Lima & Dill 1990; Biro et al. 2004, 2006; Dmitriew 2011). Eltérő kimenetelek is ismertek: (i) a csökkent aktivitású egyedek megfelelő táplálékellátottság mellett akár nagyobbak és „kövérebbek" is lehetnek (Johansson & Andersson 2009), (ii) a méret-limitált ragadozók jelenlétében éppen a gyorsabb növekedés és nagyobb testméret segíti a túlélést (Urban 2007, 2008; Bell et al. 2011), és (iii) a prédapopuláció ritkulása is vezethet a túlélők jobb táplálékellátottságán keresztül gyorsabb növekedéshez és megnövekedett testmérethez (Grether et al. 2001; Arendt & Reznick 2005). A háromtüskés pikó esetében például a ragadozók jelenléte gyorsabb növekedést és nagyobb testméretet eredményez (Bell et al. 2011; Frommen et al. 2011), hiszen a faj rendkívül hatékony mellkasi tüske – oldalpajzs – háti tüske funkcionális egységgel bír, ami a legtöbb ragadozót méret-limitálttá teszi (Reimchen 1983). A kilenctüskés pikó tüskéi azonban jóval gyengébbek (Hoogland et al. 1957) és ezért nála a ragadozók túlnyomó többségénél nem lép fel méret-limitáltság.

A fő eredmény, miszerint a ragadozó-adaptált tengeri pikók reagálnak erősebben a ragadozó-veszélyre, a kompetíció-adaptált kis tavi pikók pedig a táplálékmennyiségre támogatja de Jong (2005) elképzelését a fenotipikus plaszticitásról, mint szelekció alatt álló kvantitatív tulajdonságról. Ebben az interpretációban nem csak a tulajdonság állapota, hanem a tulajdonság plaszticitásának mértéke is fontos része a lokális adaptációnak. A plaszticitásbeli populációs eltérések habitat-függése a természetes szelekció szerepére utal (Clarke 1975; Endler 1986; Schluter & Nagel 1995; McGuigan et al. 2005). Sőt, a populáción belüli mintázat is a plaszticitás adaptációbeli szerepét erősíti: a faj testméret-evolúciójában feltehetőleg fontosabb nem, a nőstények (Herczeg et al. 2010b, 2012) plasztikus válaszai erősebbek voltak, mint a hímekéi. Az eredmények jól illeszkednek a populációs összehasonlításoknál kirajzolódott képhez (4.1): az izolált kis tavi pikók a maximális növekedés érdekében kevéssé reagálnak a ragadozó okozta veszélyre, a többlet táplálékot pedig jobban hasznosítják, mint a túlélést célzó tengeri fajtársaik.

4.2.1.2.2 Energiaraktárak

A ragadozó kezelés kizárólag a belső szerv mentes testtömegre volt hatással, habitattól függő mértékben (Välimäki, Herczeg, Merilä kézirat; Välimäki 2012). A kis tavi pikók relatív testtömege magasabb volt a tengeri pikókénál, ugyanakkor a ragadozók jelenléte csak a kis tavi pikóknál okozott enyhe relatív testtömeg csökkenést (28/a. ábra). A belső szerv-mentes testtömegben megfigyelt ivari dimorfizmus szintén habitat-függést mutatott: a hímek magasabb relatív testtömeggel bírtak, mint a nőstények, és a dimorfizmus kifejezettebb volt a kis tavakban, mint a tengeri populációkban (28/b. ábra). A relatív testtömeget befolyásolta még a táplálék kezelésünk is, a magas táplálék kezelésben fejlődő halak relatív testtömege

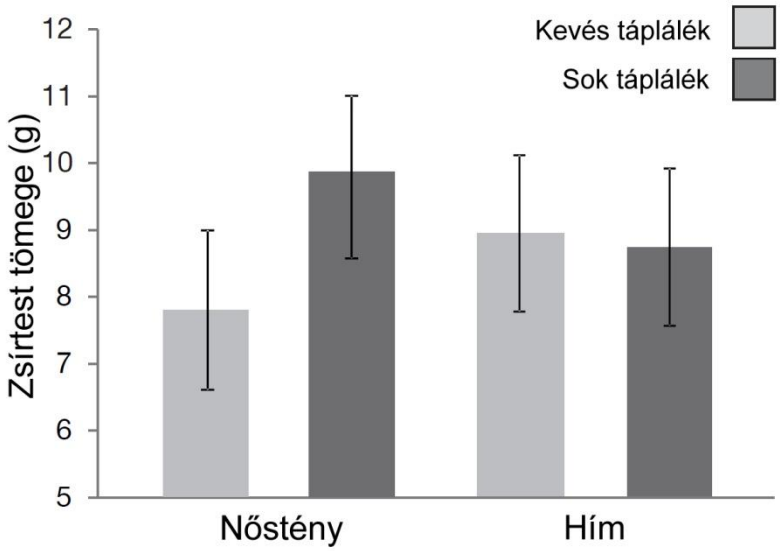
magasabb volt habitattól és ivartól függetlenül. A táplálék kezelés hatása a zsírtest relatív tömegre ivarfüggő volt, a nőstények zsírtestjei nagyobbak voltak *ad libitum* táplálék mellett, a hímeknél viszont nem volt ilyen hatás (29. ábra). A relatív májtömegnél csak egy meglepő mintázatot kaptunk, a magas táplálékellátottság mellett fejlődő pikók kisebb májat fejlesztettek.



28. ábra. Kettős interakciók hatása kilenctüskés pikók belső szerv mentes testtömegére (korrigált átlag ± standard hiba) egy tengeri és izolált kis tavi populációkon végzett, ragadozó jelenlét/hiányon és alacsony/magas taplálékellátottságon alapuló faktoriális *common garden* kísérlet alapján. A) habitat × ragadozó, B) habitat × ivar hatások.

A ragadozók okozta kockázat sok halfajnál alakít ki *trade-off* viszonyt a növekedés és az energiaraktározás között (Sogard 1997; Biro et al. 2006). Az energiaraktárakra kifejtett negatív hatást bizonyították az észak-amerikai süllő (*Stizostedion vitreum*), a pisztrángsügér (*Micropterus salmoides*) és a tavi szaibling (*Salvelinus alpinus*) esetében is (Pratt & Fox 2002; Garvey et al. 2004; Laakonen

2006). A hatás nem volt minden esetben általános, például a tavi szaiblingnél a relatív testtömeg csökkent, a zsírtartalom viszont nőtt ragadozó jelenlétében (Laakonen 2006). Mi azt vártuk, hogy a ragadozó-adaptált tengeri kilenctüskés pikók erősebb választ mutatnak majd a ragadozó kezelésünkre, mint a kis tavi pikók, de ellenkező hatást találtunk, a tengeri pikók raktárait nem befolyásolta a ragadozók jelenléte, míg a kis tavi pikók csökkentették a relatív testtömegüket a ragadozó kezelésben. Elképzelhető, hogy a tengeri pikók energiaraktározási stratégiája a ragadozók függvényében kanalizálódott, mivel folyamatosan nagy ragadozó nyomás alatt evolválódtak (Crispo 2007), a kis tavi pikók viszont a ragadozók hiánya ellenére megőrizték a plaszticitásukat. A táplálék kezelés szempontjából a többlet táplálék pozitív hatását feltételeztük az energiaraktárakra. Ezt a relatív testtömeg és zsírtest mintázatok támogatták, a máj relatív tömege viszont csökkent a magas táplálék kezelés hatására. Más vizsgálatokban a máj tömege pozitív összefüggést mutatott a táplálék mennyiségével (Allen & Wootton 1982; Pelletier et al. 1994; Ali & Wootton 1999), ami logikus is a máj szénhidrát és zsír raktár szerepét tekintve (Chellapa et al. 1989, 1995). A mi vizsgálatunkban talált negatív májtömeg-táplálékmennyiség összefüggésre jelenleg még nincs magyarázatunk.



29. ábra. A táplálék kezelés hatása különböző ivarú kilenctüskés pikók relatív zsírtest tömegére (korrigált átlag ± standard hiba).

Az egyetlen habitat-függő plasztikus válasz, ami megfelelt a várakozásainknak a relatív testtömegben volt megfigyelhető, ugyanis a táplálékmennyiség pozitív hatása a kis tavi pikóknál erősebb volt, mint a tengerieknél. Ez összecseng az előző fejezetben tárgyalt testméret mintázattal, mintegy azt támogatva: a kis tavi pikók nem csak nagyobbra nőttek a többlet energiát kihasználva, hanem relatíve nehezebb testük is lett. Megjegyzendő, a relatív testtömeg pontos jelentése nem egyértelmű, jelenthet magasabb szénhidrát és zsír raktárakat a vázizomzatban és/vagy tömegesebb izomzatot és csontozatot is, tehát a pontos interpretáció ennél a változónál problematikus (lásd még: 4.1.3.3).

4.2.1.3 Agy és érzékszervek

Az agy plaszticitására rengeteg a példa különböző neuroanatómiai szinteken, az egyedfejlődés különböző állapotaiban és sokféle taxonnál (Diamond et al. 1966; Kempermann et al. 1997; Tramontin & Brenowitz 2000; Zupanc 2001). Az elmúlt évtizedekben a kísérletes megközelítésnek köszönhetően egy sor abiotikus és biotikus környezeti változót azonosítottak, melyek mind befolyásolják az agy fejlődését (van Praag et al. 2000; Mohammed et al. 2002). A legegyszerűbb környezeti változatosság, például néhány kő berakása a nevelő tartályokba mérhető *cerebellum*-növekedéssel járt szivárványos pisztrángnál (*Oncorhynchus mykiss*; Kihslinger & Nevitt 2006), sőt, egy közelrokon fajnál a fizikai környezet változatosságának pozitív hatását figyelték meg a sejtosztódás intenzitására a *telencephalon*-ban (Lema et al. 2005). Ökológiailag releváns környezeti faktorok interakcióját, illetve olyan biotikus környezeti faktorok hatását, mint például a predáció vagy a kompetíció az agy fejlődésére még alig vizsgálták (Gonda et al. 2010; Trokovic et al. 2011b).

Az érzékszervek adaptív fenotipikus plaszticitásáról kevés információ áll rendelkezésünkre (Dangles et al. 2009). A legtöbb ilyen vizsgálat gerinctelenekkel foglalkozik (Opstad et al. 2004; Cronin et al. 2010; Merry et al. 2011), gerincesek közül főleg a halak látása került előtérbe (Fuller et al. 2005; Chapman et al. 2010; Smith et al. 2011). A halakra és vízi kétéltűekre jellemző mechanoreceptoros oldalvonalszerv (Dijkgraaf 1963) fajon belüli változatosságának feltárása még gyerekcipőben jár (4.1.4.2; Michel et al. 2008, Wark & Peichel 2010; Trokovic et al. 2011a), a tulajdonság adaptív fenotipikus plaszticitásáról pedig semmit sem tudunk.

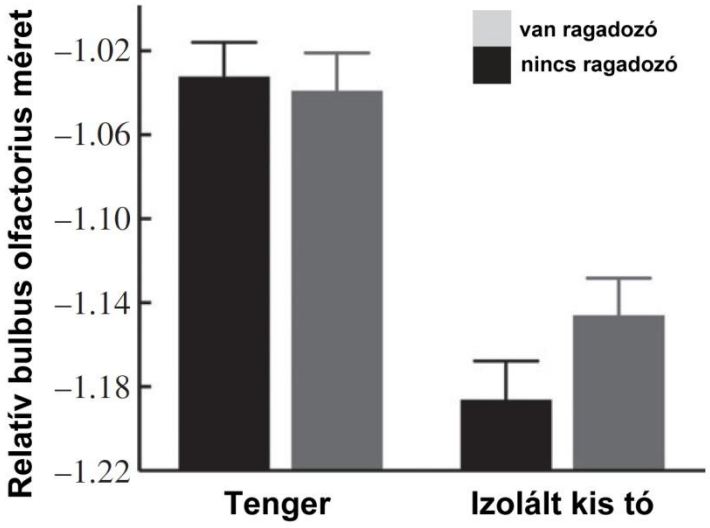
A populációs összehasonlításainkban (4.1.4; Gonda et al. 2009a, 2011; Trokovic et al. 2011a) mind az agy, mind a komplex oldalvonalszerv felépítése kapcsán találtunk habitat-függő, azaz a természetes szelekció szerepét támogató különbségeket. Ezekben a tág értelembe véve is pionír vizsgálatokban nem tudtunk pontos ok-okozati összefüggéseket bizonyítani, már a fajon belüli adaptív evolúció létének támogatása is nagy előrelépést jelentett (Gonda et al. 2013). A természetben megfigyelt fajon belüli változatosság mechanizmusainak mélyebb megértését segíthetik az adott tulajdonságok predáció- és táplálékellátottság-indukálta fenotipikus plaszticitásának és a plaszticitás esetleges populáció- vagy habitat-alapú eltéréseinek vizsgálatai.

4.2.1.3.1 Agyméret és az agyterületek mérete

A ragadozók szaganyagainak jelenléte habitat-függő plasztikus választ indukált a *bulbus olfactorius*-ban, illetve habitattól és ivartól független plasztikus választ a *hypothalamus*-ban (Gonda et al. 2012). Bár az izolált kis tavi pikók általánosan kisebb *bulbus olfactorius*-t fejlesztettek, mint a tengeri pikók, a ragadozók szaganyagaira csak a kis tavi pikók reagáltak nagyobb *bulbus olfactorius*

fejlesztésével (30. ábra). A ragadozó kezelésnek negatív hatása volt a *hypothalamus* fejlődésére. A táplálék kezelésnek nem volt szignifikáns hatása.

A fő eredményünknek az tekinthető, hogy sikerült bizonyítani a ragadozók okozta veszély (fizikai érintkezés nélkül) agystruktúra formáló hatását. Erre eddig egyetlen kétéltűnél volt példa (Gonda et al. 2010; Trokovic et al. 2011b). A *bulbus olfactorius* (szaglóközpont) esetében a hatás habitat-függő volt, ami adaptív evolúcióra utal (Clarke 1975; Endler 1986; Schluter & Nagel 1995; McGuigan et al. 2005). Elsőre meghökkentő módon, a ragadozó nélkül evolválódó kis tavi pikók mutattak csak plasztikus választ a ragadozókra. Amennyiben azonban figyelembe vesszük a tengeri pikók általánosan nagyobb *bulbus olfactorius* méretét, már érthetőbb a mintázat; feltehetően az állandó és magas predációs nyomás alatt élő tengeri pikóknál a lehető legnagyobb *bulbus olfactorius* evolválódott, miközben a plaszticitásra való képesség megszűnt a genetikai asszimiláció következtében (Crispo 2007). A kis tavi pikóknál a *bulbus olfactorius* kisebb, de a plaszticitásra való képesség jelen van. Összességében, a központi idegrendszer szaglóközpontja fontos szereppel bírhat a ragadozók elleni viselkedésben, hiszen a ragadozók jelenléte mind evolúciós, mind ontogenetikus skálán is a nagyobb méretét indukálja. Ugyanakkor a tény, miszerint egy adott tulajdonság plaszticitása a tulajdonság csökkenésével párhuzamosan jelenik meg (a tengeri populáció tekinthetőek ősinek, a kis tavi populációk pedig leszármazottnak) egyelőre nem megmagyarázható, és további vizsgálatokat igényel.



30. ábra. Habitat-függő ragadozó kezelés hatás a kilenctüskés pikók *bulbus olfactorius* fejlődésére (korrigált átlag ± standard hiba).

A ragadozó kezelés általánosan csökkentette a *hypothalamus* méretét. Ennek az agyterületnek rengeteg ismert funkciója van (Kotrschal et al. 1998). Többek között befolyásolja a szaporodási (White & Fernald 1993) és táplálkozási viselkedést (Kulczykowska & Sánchez Vázquez 2010). Az adataink alapján lehetetlen egyértelműen rámutatni a ragadozó nyomás és a *hypothalamus* méret közti pontos

mechanizmusra, de mivel ismert a ragadozók táplálkozási aktivitásra kifejtett negatív hatása (Biro et al. 2004, 2006; Kulczykowska & Sánchez Vázquez 2010) és a kísérletünkben mi is megfigyeltük a ragadozó kezelés negatív hatását az agresszióra és kockázatvállalásra (4.2.1.4; Herczeg & Välimäki 2011), feltételezhető, hogy a *hypothalamus* méretének csökkenése valahogy összefügg a viselkedési aktivitás csökkenésével.

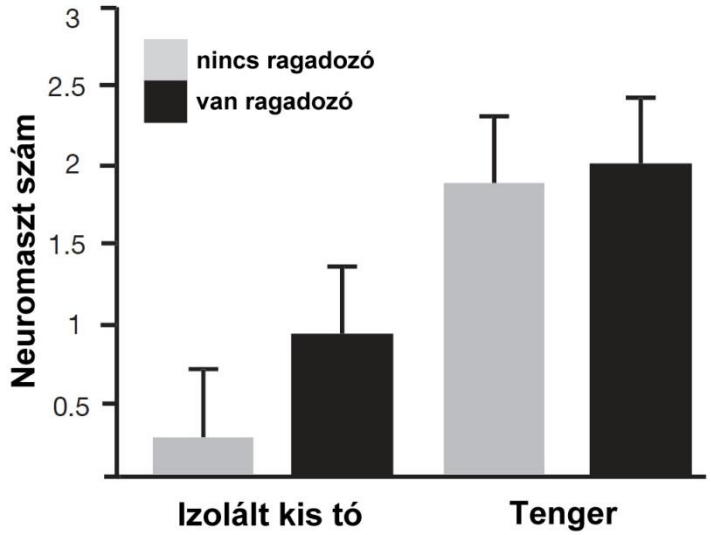
Ivar-függő fenotipikus plaszticitást egyedül a *bulbus olfactorius*-nál tapasztaltunk, a nőstény pikók nagyobb szaglóközpontot fejlesztettek *ad libitum* táplálékellátottság mellett, mint a hímek, ám ez a különbség eltűnt alacsony táplálékellátottságnál (Herczeg et al. 2014). A mintázat pontos magyarázata nem ismert, de az ivar-függő fenotipikus plaszticitás a szaglóközpontban további vizsgálatokat érdemel.

4.2.1.3.2 Oldalvonalszerv

Az analíziseink a kilenctüskés pikók oldalvonalszervét alkotó egyes oldalvonalak nagy fajon belüli változatosságát bizonyították, a habitat, az ivar, a kezelések és ezek változatos interakcióinak szerepével együtt (Välimäki, Herczeg, Trokovic, Merilä kézirat; Välimäki 2012). Mivel az egyes oldalvonalak specifikus funkciója ismeretlen, csak a két, habitat-függő plaszticitást mutató oldalvonalnál megfigyelt mintázat ismertetésére térek ki. A kopoltyúfedőn található felszíni neuromasztok számát a ragadozó kezelés habitatonként eltérően befolyásolta. A tengeri pikók neuromasztjainak száma meghaladta a kis tavi pikókét, de csak ez utóbbiak reagáltak a ragadozók jelenlétére a neuromaszt számuk növelésével (31. ábra). A törzs anterior részén lévő felszíni neuromasztok esetében egy komplex habitat × ivar × ragadozó kezelés × táplálék kezelés interakciót találtunk. Nemenként vizsgálva a mintázatot a következő kép bontakozott ki: (i) a tengeri pikóknak több neuromasztjuk volt mint a kis tavi fajtársaiknak, (ii) a tengeri nőstények neuromaszt száma alacsony táplálékellátottság mellett pozitívan, magas táplálékellátottság mellett pedig negatívan függött össze a ragadozók jelenlétével, (iii) a kis tavi hímek neuromaszt száma nőtt a ragadozók szaganyagának hatására és (iv) a kis tavi hímeknek magasabb volt a neuromaszt számuk magas, mint alacsony táplálékellátottság mellett.

Az eredmények demonstrálják az oldalvonalszerv fajon belüli változatosságát (az elővizsgálathoz [4.1.4.2; Trokovic et al. 2011a] hasonlóan), elsőként bizonyítják az oldalvonalszerv környezeti faktorok által indukált fenotipikus plaszticitását, és a plaszticitás habitat- és ivar-függését. Az érzékszervek plaszticitását gyakran feltételezik, de a hipotézis tesztelése még gyerekcipőben jár (Dangles et al. 2009). Az oldalvonalszerv szerepe a ragadozóellenes viselkedésben régóta ismert (Blaxter & Fuiman 1990; McHenry et al. 2009). A vizsgálatunk bizonyította, hogy a ragadozók szaganyagai (aktuális ragadozó-préda találkozás vagy vizuális ingerek nélkül) a kilenctüskés pikók neuromaszt számának növekedését indukálják. A neuromasztok számának növekedése az adott oldalvonal érzékelő-képességének növekedésével jár

együtt (Coombs et al. 1988; Yoshizawa et al. 2010; Yoshizawa & Jeffrey 2011), a jobb felbontóképességű oldalvonalszerv pedig segítheti a ragadozók elkerülését. Ezek alapján jogosan tételezhető fel a ragadozó-indukálta plaszticitás adaptivitása. Mivel az oldalvonalszerv felépítése és plaszticitása egyaránt habitat-függő mintázatokat mutatott *common garden* körülmények között, feltehető, hogy az egyes oldalvonalak neuromasztjainak száma és a plaszticitásuk egyaránt adaptív evolúció eredményeként mutat populációs szétválást (Clarke 1975; Endler 1986; Schluter & Nagel 1995; McGuigan et al. 2005). A változatos interakciókat interpretálni nem könnyű, de az oldalvonalszerv nagyfokú változékonyságára mindenképpen felhívják a figyelmet.



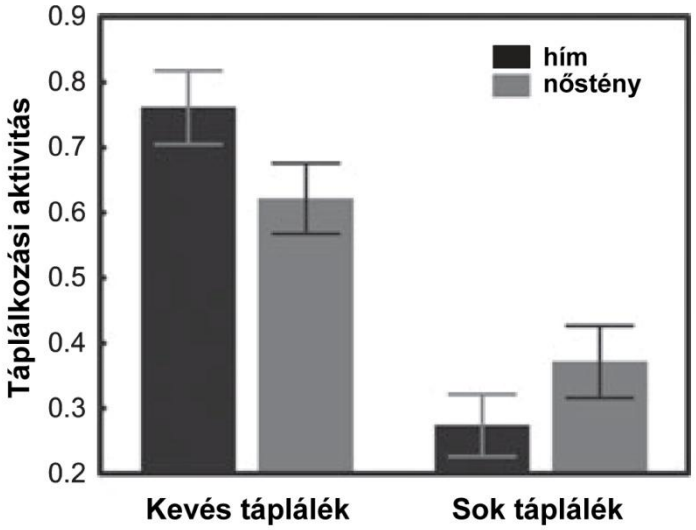
31. ábra. Habitat-függő ragadozó kezelés hatás a kilenctüskés pikók operkuláris neuromaszt számára (korrigált átlag ± standard hiba).

A kopoltyúfedő neuromasztjaiban megfigyelt plaszticitás ellentmondott a várakozásainknak: a tengeri pikóknak ugyan több neuromasztjuk volt, mint a kis tavi fajtársaiknak, de ragadozó indukálta (pozitív) plaszticitás csak a ragadozó halak hiányában evolválódott kis tavi pikóknál volt megfigyelhető. Ez a mintázat megegyezik az előző fejezetben (4.2.1.3.1; Gonda et al. 2012) tárgyalt, az agy szaglóközpontjánál megfigyelttel. A magyarázatunk is megegyezik, a magas predációs nyomás alatt élő tengeri pikók feltehetően a maximális számú neuromasztot fejlesztik, és az idevezető fejlődési út már kanalizálódott (Crispo 2007), a kis tavi pikók pedig megőrizték (kifejlesztették?) a plaszticitásra való képességet.

4.2.1.4 Viselkedés

Három vizsgált viselkedési változónál (táplálkozási aktivitás, kockázatvállalás táplálkozási kontextusban, agresszió) találtunk plaszticitást (Herczeg & Välimäki 2011). A táplálkozási aktivitás ivar-függő plaszticitást mutatott a táplálék kezelés hatására (32. ábra). Alacsony táplálékellátottság mellett megnőtt mindkét ivar

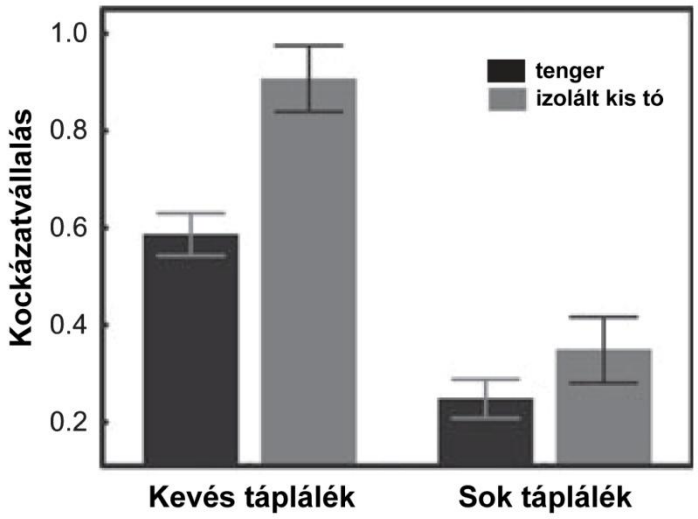
táplálkozási aktivitása, de a kezelések közötti eltérés a hímeknél magasabb volt. A kockázatvállalásnál habitat-függő táplálék kezelés hatást találtunk: alacsony táplálékellátottság mellett megnőtt a kockázatvállalás, de a hatás erősebb volt a kis tavi pikóknál (33. ábra). A ragadozó kezelés egységesen csökkentette a kockázatvállalást és az agressziót.



32. ábra. Ivar-függő táplálék kezelés hatás a kilenctükés pikók táplálkozási aktivitására (átlag ± standard hiba).

Mint a többi plaszticitásos vizsgálatunknál, itt is a habitat-függő plaszticitásra voltunk a leginkább kíváncsiak. Több halfajnál is megfigyeltek populációs szétválást plaszticitásban, általában a kísérletesen manipulált környezeti faktorhoz (pl. ragadozók jelenléte, vízáramlás) a természetben alkalmazkodott populációk mutattak nagyobb plaszticitást (Magurran 1990b; Rodd & Sokolowski 1995; Salonen & Peuhkuri 2007). Hasonló mintázatokat találtak például vízibolháknál (Cladocera) is (de Meester 1993, 1996). A mi esetünkben az érzékelt ragadozó kockázat nem okozott habitat-függő plaszticitást, a táplálékhiány azonban nagyobb kockázatvállalás-növekedést okozott az izolált kis tavi, mint a tengeri pikóknál. Táplálékhiányos szituációban más halaknál is megnövekedett a kockázatvállalás (pl. Biro et al. 2005). A kockázatvállalás-növekedés habitat-függését az eddigi eredményeink alapján könnyű interpretálni. A méret-független ragadozó nyomás az intenzív növekedés és a nagy testméret ellen szelektál (Blanckenhorn 2000; Biro et al. 2004, 2006), míg az interspecifikus kompetíció általában a méretváltozás ellen hat (Wilson 1975; Lomolino 1985; Simberloff et al. 2000). Az izolált kis tavakban mindkét hatás minimális, és ezekben a populációkban sokáig növekedő, óriás méretű pikókat találunk (Herczeg et al. 2009a, 2012). Feltételezésünk szerint, mivel a kis tavakban az élettartam majdnem kétszerese a többi habitat-típusban megfigyeltnek (Herczeg et al. 2009a), és a faj fekunditásának méret-függése is ismert (Heins et al. 2003, 2005; Herczeg et al. 2010b; Ab Ghani et al. 2012), az óriás méret fekunditás-előnye meghaladja az elnyújtott növekedés költségeit, beleértve az időben kitolt

ivarérés hátrányát. Ezt a feltételezést matematikai modelljeink is megerősítették (Aikio et al. 2013). Ezért érthető, hogy a minél nagyobb testméret elérésére törekvő kis tavi pikók nagyobb kockázatot vállaltak táplálékhiány esetén, mint az alapvetően a túlélésük maximalizálására koncentráló tengeri fajtársaik.



33. ábra. Habitat-függő táplálék kezelés hatás a kilenctükés pikók kockázatvállalására (átlag ± standard hiba).

Általában a fenotipikus plaszticitás képességének költségei (DeWitt et al. 1998; Auld et al. 2010) miatt a plaszticitást kiváltó környezeti tényező megszűntekor a plaszticitás képességének eltűnését várjuk. A viselkedési plaszticitás aránylag alacsony költségei miatt azonban nehéz megjósolni a ragadozó-indukálta viselkedési plaszticitás megváltozását a predációs nyomás csökkenésekor, egyes esetekben a ragadozók felismerésének képessége megmarad, máskor lassan csökken (Fong et al. 1995). Ismertek esetek ahol a ragadozók kiváltotta viselkedési válaszok igen hosszú ideig megmaradtak (Coss 1999; Blumstein 2006; Lahti et al. 2009). Ennek oka lehet például a ragadozó elleni válasz más fontos funkciója, kritikus idegrendszeri folyamatokhoz kapcsolódása, vagy ha a válasz komplex genetikai interakciók eredménye (Coss 1999; Magurran 1999; Blumstein 2006). A mi esetünkben mindenesetre a szimpatrikus ragadozó halaktól mentes környezetben evolválódó kis tavi pikók a nagy ragadozó nyomás alatt evolválódó fajtársaikhoz hasonlóan felismerték a csapó sügér szaganyagait, és azok jelenlétében a tengeri pikókhoz hasonló mértékben csökkentették a kockázatvállalásukat és agressziójukat.

4.2.2 Társas élet hatásai

A sok taxonnál előforduló csoportos életmód nagy tudományos figyelmet kapott a múltban, és kap ma is. A jelenség kialakulásának ultimális okai és költség-nyereség viszonyai alaposan kutatottak (pl. Pitcher & Parrish 1993; Krause & Ruxton 2002). A csoportos élet fő nyereségének a predáció okozta mortalitás csökkentését tekinthetjük, ami több mechanizmuson, mint például a megnövekedett éberségen, a

„felhígult” ragadozó okozta kockázaton vagy a ragadozó összezavarásán alapulhat. Előnyös lehet még a táplálék vagy a szaporodó partner megtalálásában is (Pitcher et al 1982; Magurran & Pitcher 1983; Höglund & Alatalo 1995). A nyereségekhez képest a csoportos élettel járó költségek kevésbé kutatottak. Költségként jelentkezhet a véges források megosztása és a következményképpen jelentkező megnövekedett kompetíció (Pitcher & Parrish 1993; Krause & Ruxton 2002), illetve egyes ragadozók kimondottan preferálhatják a csoportokat (Krause & Godin 1995; Botham & Krause 2005; Ioannou & Krause 2008). A parazitafertőzések szempontjából kevésbé egyértelmű a helyzet, a jól terjedő paraziták ellen jó a nagy csoport a paraziták „felhígulása” miatt, de az érintéssel terjedő parazitáknál kimondottan káros a csoportos életmód (Poulin & Fitzgerald 1989; Côté & Poulin 1995; Poulin 1999).

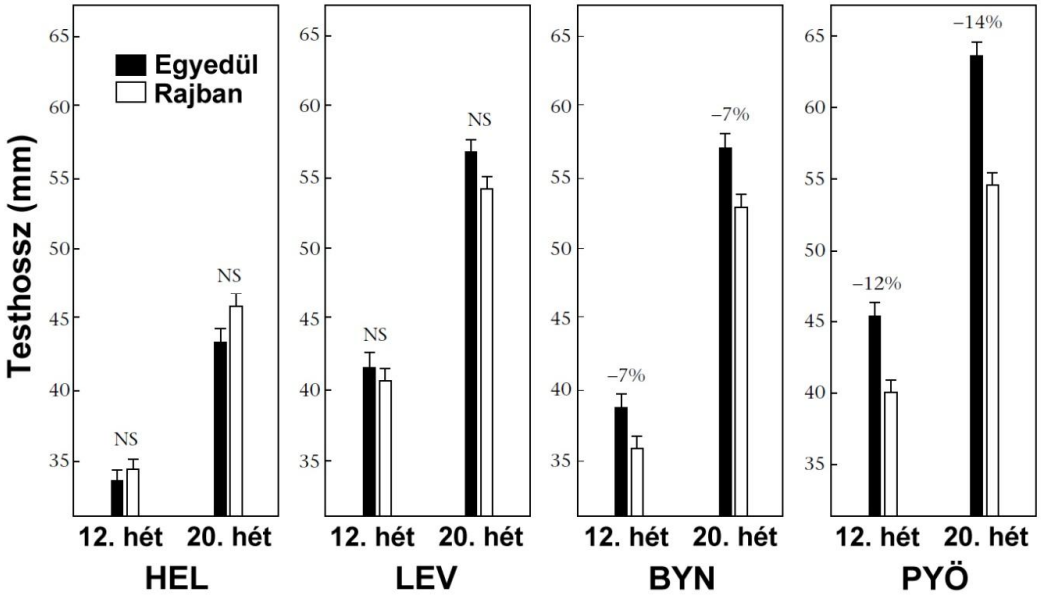
A legkevésbé ismert feltételezett káros hatás a szociabilitás direkt költsége, ami független az eddig felsorolt ökológiai tényezőktől és pusztán a társas interakciókból (pl. agresszió okozta stressz) ered. Ahol a csoportos életmód előnyös, ott feltételezhetően a szelekció e pusztán az egyedek viselkedésén alapuló költség ellen hat. Erre közvetett bizonyíték lehet az agresszió és a csoportalkotási tendencia közötti negatív kovariáció (Magurran & Seghers 1991). A különböző habitatokban élő, különböző életmenet és viselkedési stratégiákat követő kilenctüskés pikók kiváló modellek a szociabilitás direkt költségének vizsgálatára, amely vizsgálat ugyanakkor értékes adatokkal szolgálhat a kilenctüskés pikók adaptív szétválásának megértéséhez is.

Két tengeri és két izolált kis tavi populáció fogságban szaporított generációit vizsgáltuk *common garden* körülmények között két kezelést alkalmazva: a halak felét egyedül, fajtársaktól elzárva, a másik felét pedig rajban tartva neveltük fel. Vizsgáltuk a növekedésüket (Herczeg et al. 2009c) és az agyuk méretét és struktúráját (Gonda et al. 2009b).

4.2.2.1 Testméret

Mint azt korábban már tárgyaltam, a testméret és a növekedés kiemelt fontosságú rátermettséget befolyásoló életmenet komponensek (Peters 1983; Roff 1992; Stearns 1992; Dmitriew 2011), előfordul, hogy közvetlenül a rátermettség becslésére is használják őket (Bolnick & Lau 2008). Jelen vizsgálatban (Herczeg et al. 2009c) arra voltunk kíváncsiak, hogy egy ökológiai költségektől mentes környezetben befolyásolja-e pusztán a fajtársak jelenléte a különböző habitatokból származó kilenctüskés pikók testméretét?

Eredményeink szerint a fajtársak jelenléte negatívan befolyásolta az izolált kis tavi pikók növekedését *ad libitum* táplálékkínálat, valamint a ragadozók, paraziták és a szaporodás költségeinek hiánya mellett, míg a tengeri pikóknál nem találtunk negatív hatást (34. ábra). A növekedési deficit az egyik kis tavi populációnál elérte a 14%-ot. Vizsgáltuk a rajképzési hajlamot is, és az eredményeink szerint stresszes környezetben az összes populáció egyedei hasonló affinitással képeztek rajokat.



34. ábra. A csoportos élet hatása a növekedésre (átlag + 95% konfidencia intervallum) tengeri (HEL, LEV) és izolált kis tavi (BYN, PYÖ) kilenctüskés pikóknál. NS = nem szignifikáns. A negatív százalékos értékek a kezelés szignifikáns mivoltakor a csoportos élet növekedésre kifejtett negatív hatását számszerűsítik. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

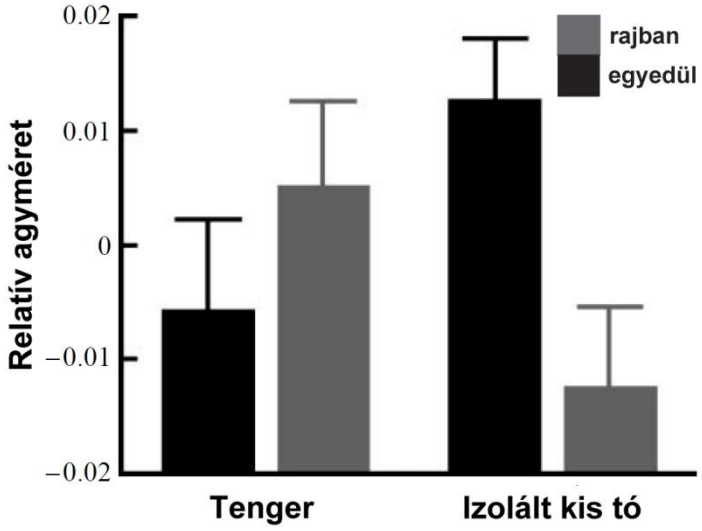
Vizsgálatunkban bizonyítottuk a szociabilitás direkt költségét, ami tisztán a fajtársak közötti szociális interakciókból ered. Ráadásul a *common garden* körülmények között megfigyelt mintázat ismét erős habitat-specificitást mutatott, ezzel a természetes szelekció szerepére utalva (Clarke 1975; Endler 1986; Schluter & Nagel 1995; McGuigan et al. 2005). A kísérletünkben közvetlen rátermettség-változókat nem mértünk, de ismeretes a nőstények fekunditásának méret-függése (Heins et al. 2003, 2005; Herczeg et al. 2010b; Ab Ghani et al. 2012) és a territorialitáson alapuló hím szaporodási viselkedés sikere is feltehetően méretfüggő. A kísérlet 20 hete alatt a pikók elérték az ivarérett testméretet. Tehát az izolált kis tavi populációkban – maximális felvehető energiamennyiség mellett és ökológiai kényszerek nélkül – megfigyelt 7-14% növekedésdeficit negatív hatása a rátermettségre biztosra vehető. Az eddigi eredményeink alapján ez nem is meglepő, hiszen bizonyítást nyert a kis tavi kompetíció-adaptált fenotípus jelenléte, amely hosszú idő alatt óriási méretre nő (Herczeg et al. 2009a, 2012) akár az ivarérés késleltetése árán is (Herczeg et al. 2009a; Ab Ghani et al. 2013), minden energiáját a növekedésbe fekteti (Välimäki, Herczeg, Merilä kézirat) és emellett agresszív és kockázatvállaló (Herczeg et al. 2009b; Herczeg & Välimäki 2011). A rajképzési hajlam eltérhet populációk között a predációs nyomás függvényében (pl. Brown & Warburton 1997) és heritábilitást is mutat (Breden et al. 1987; Magurran 1990b). Ezért volt meglepő a populációk közötti rajképzési hajlambeli eltérés hiánya. Mindenesetre a természetben sokféle ragadozó halfajjal együtt élő tengeri pikóknál sokkal gyakoribb a rajképzés (saját megfigyelés) és ezért logikusnak tűnik, hogy az ilyen populációkban a szociabilitás direkt költsége minimális, szemben az izolált kis

tavi pikókkal, akik a rajképzésből nem profitálhatnak annyit, mint amennyi költségett a megnövekedett agresszió okoz nekik.

4.2.2.2 Agyméret és az agyterületek mérete

A szociális környezet komplexitását tartják a főemlősök meglepően nagy agya mögött álló környezeti faktornak (Dunbar & Schultz 2007), és ez az elmélet támogatást nyert egyéb emlősfajoknál is (Perez-Barberia et al. 2007). Kísérletes vizsgálatok a szociális környezet hatásával kapcsolatban meglehetősen ritkák, és főleg az emlősökre és madarakra szorítkoznak (Fowler et al 2002; Lipkind et al. 2002; Adar et al. 2008). Jelen vizsgálatunkban (Gonda et al. 2009b) arra kerestük a választ, hogy okoz-e a szociális környezet változatossága változást az agyméretben és –struktúrában, illetve a hatás eltér-e a ragadozó-adaptált tengeri és a kompetíció-adaptált izolált kis tavi kilenctüskés pikók között?

A kezelésünknek habitat-függő és -független hatásai egyaránt voltak. Az izolált kis tavi pikók relatív agymérete csökkent a társas kezelés hatására, míg a tengeri pikóknál inkább egy ellentétes trend volt megfigyelhető (35. ábra). A populációs eredettől függetlenül, a csoportosan tartott pikóknál relatíve nagyobb *tectum opticum*, a magányos halaknál pedig relatíve nagyobb *bulbus olfactorius* fejlődött.



35. ábra. A csoportos élet hatása az agyméretre (korrigált átlag ± standard hiba) tengeri és izolált kis tavi kilenctüskés pikóknál.

Az eredményeink elsőként bizonyították a szociális környezet szerepét az agyfejlődésben halaknál, illetve elsőként bizonyítottunk populációs eltérést az agy plaszticitásában. A habitat-függő mintázat a természetes szelekció szerepét valószínűsíti (Clarke 1975; Endler 1986; Schluter & Nagel 1995; McGuigan et al. 2005) és tökéletesen kapcsolódik az előző fejezetben (4.2.2.1) tárgyalt növekedési mintázattal: a rajban tartott izolált kis tavi pikóknál fejlődési deficit figyelhető meg. Megjegyzendő, hogy az analízisünkben az agyméret korrigálva volt a testméretre, tehát az itt kimutatott deficit az általános növekedési deficiten felül jelentkezett. A

magyarázat is megegyezik a növekedési mintázatoknál tárgyalttal; a nagy növekedési erélyű, agresszív kis tavi fenotípus számára a szociabilitás direkt költsége még *ad libitum* táplálékkínálat és zéró ökológiai költségek mellett is fejlődési deficitet okoz. Különösen erős költségre utal a jelen eredmény, hiszen a kommunikáció és egyéb viselkedési interakciók megjelenése a rajban tartott csoportnál elvileg nagyobb agyat is eredményezhetne (Fowler et al. 2002; Lipkind et al. 2002; Adar et al. 2008), amire csak a tengeri pikóknál találtunk is egy gyenge trendet.

Mivel az idegrendszer az egyik legköltségesebb fejlesztésű és fenntartású szövet (Aiello & Wheeler 1995; Kotrschal et al. 2013), az agyterületek relatív mérete jól becsli az adott kontextusban betöltött szerepük fontosságát (Kotrschal et al. 1998; Kihslinger et al. 2006; Lisney et al. 2007). Az alkalmazott kezeléseinkből adódóan a magányosan tartott halak a környezetükről szinte kizárólag olfaktórikus információkon keresztül szerezhettek információt, a rajban tartott halak számára viszont a vizuális ingerek is fontosak voltak. Így aztán nem meglepő, hogy a magányos halaknál a szaglóközpont, a csoportosan tartott halaknál viszont a látóközpont volt relatíve nagyobb. A szagló- és látó-központok közötti evolúciós *trade-off* viszony emlősöknél ismert (Barton et al. 1995; Barton & Harvey 2000), halaknál viszont nem (Van Staaden et al. 1995; Huber et al. 1997). A mi esetünkben viszont egy egyedfejlődés alatti *trade-off*-ról van szó, ami feltehetőleg több agyterület viszonylatában és sok taxonnál lehet egy kritikus fejlődési mechanizmus. A kérdés további vizsgálatával a mechanizmus szintjén találhatnánk választ az agy evolúció egyik fő kérdésére, miszerint a különböző funkcióval bíró agyterületek egymástól függetlenül (*mosaic evolution*; Barton & Harvey 2000), vagy éppen ellenkezőleg, erős fejlődési és genetikai kényszerek alatt közösen (*concerted evolution*; Finlay & Darlington 1995; Finlay et al. 2001) változnak?

4.2.3 Összegzés

A második, fenotipikus plaszticitással foglalkozó fejezetben sok releváns tulajdonságnál találtunk az első fejezetben fontosnak talált környezeti változók (predáció, kompetíció) változatosságának laboratóriumi imitációjára mutatott plasztikus választ. A detektált mintázatok sokszínűek voltak, az előzetes várakozásainknak csak részben feleltek meg. Feltételeztük, hogy egy adott környezeti tényezőhöz (pl. predáció) való alkalmazkodás része a tényező változatossága által kiváltható megnövekedett plaszticitás is. Erre több esetben is találtunk bizonyítékot, például a testméretnél vagy a kockázatvállalásnál. Ez a fenotipikus plaszticitás kvantitatív tulajdonságként való evolúciójára utal. Ugyanakkor ellenkező irányú összefüggésekre is fény derült: az agy szaglóközpontjának mérete, illetve egy bizonyos oldalvonalszerv neuromaszt-száma csak a ragadozóhal-mentes környezethez adaptálódott populációkban mutatott plaszticitást a ragadozó halak jelenlétének-hiányának függvényében. Mivel a ragadozóhal-adaptált populációkban találtunk nagyobb szaglóközpontot és több neuromasztot, itt feltételezhető a tulajdonság maximális kifejeződésének kanalizáció útján való fixálódása és következésképpen a plaszticitás elvesztése, de a

ragadozóhal-mentes populációkban az alacsonyabb jellegállapot mellett megőrzött (vagy megjelent?) plaszticitásra nincs magyarázatunk. Ez utóbbi eredmények értelemszerűen ellentmondanak annak a várakozásunknak is, miszerint egy stabilan hiányzó környezeti stimulusra adható plasztikus válasz a plaszticitás költségei miatt redukálódik vagy eltűnik.

A különböző jellegű tulajdonságokban eltérő erősségű plaszticitást vártunk. Ezek a várakozások megerősítést nyertek: a morfológiai tulajdonságok minimális plaszticitást mutattak, az életmenet és neurobiológiai tulajdonságoknál már sok környezet indukálta választ találtunk, míg a viselkedési tulajdonságoknál egyöntetűen magas plaszticitást találtunk. A legtöbb esetben a természetes populációkban megfigyelt mintázatok kialakításában a plaszticitás (ha jelen volt) a genetikai adaptációk irányába hatva azok hatását felerősítethette, de ellene egy esetben sem hatott, így esetünkben *countergradient variation*-ra nem találtunk bizonyítékot.

4.3 Kvantitatív genetikai háttér

A 4.1 pontban tárgyalt populációs különbségek habitat-függéséből és a mintázatok *common garden* körülmények közötti megismételhetőségéből azok genetikai meghatározottságára és a természetes szelekció szerepére a kialakulásukban következtettünk. Az ilyen következtetések megalapozottnak tekinthetőek (Clarke 1975; Endler 1986; Schluter & Nagel 1995; McGuigan et al. 2005), de semmiképpen nem bizonyítóak, hiszen az anyai hatásokat vagy a generációkon átívelően is megjelenő környezeti hatásokat egyszerű összehasonlításokkal nem lehet kizárni (Falconer & McKay 1996; Lynch & Walsh 1998). Az evolúciós interpretáció szempontjából viszont a genetikai háttér bizonyítása elengedhetetlen, hiszen a természetes szelekció csak a genetikai komponenssel is rendelkező egyedek közötti fenotípusos változatosságra kifejtett hatásával okozhat adaptív evolúciót.

Jelen fejezet célja a korábban (4.1) bemutatott tengeri és izolált kis tavi kilenctüskés pikók tulajdonságaiban megfigyelt eltérések genetikai hátterének feltárása és bizonyítása volt. Ezt három megközelítéssel kíséreltük meg: (i) reciprok hibridek és az eredeti populációk laboratóriumi generációinak összehasonlításával kerestük a populációs szétválásért leginkább felelős kvantitatív genetikai komponenseket, (ii) egy általunk tervezett új *genome scan* módszerrel kerestünk természetes szelekció alatt álló genomrégiókat, és végül (iii) *QTL mapping* módszerrel azonosítottuk a konkrét tulajdonságok változatosságáért felelős régiókat a genomban.

4.3.1 A populációs szétválás háttere

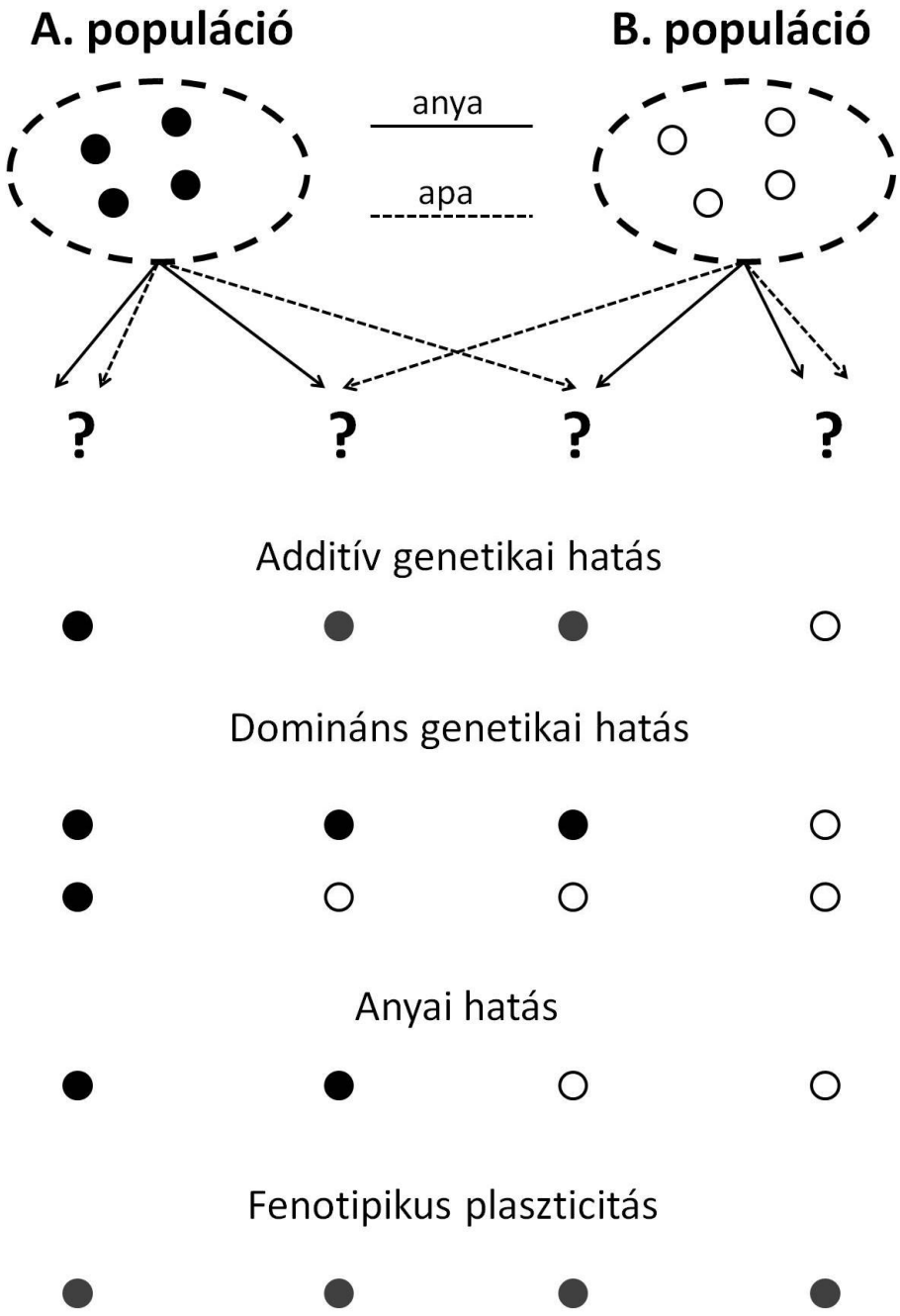
A rátermettséget leginkább befolyásoló viselkedési és életmenet tulajdonságok esetében a fajon belüli, populációk közötti változatosság jól dokumentált (pl. Stearns 1983, 1992; Roff 1992, 2002; Foster 1999; Foster & Endler 1999; Lynch et al. 1999; Quinn et al. 2000), de a populációs szétválás genetikai háttere a legtöbbször

ismeretlen. Ennek gyakorlati okai vannak, a különböző genetikai komponensek szétválasztása az anyai vagy környezeti hatásoktól problematikus, a geográfiailag elkülönülő populációk közötti reciprok hibridek analízisén alapul (Lynch & Walsh 1998), ami sok esetben nehezen kivitelezhető. Bár az optimális megközelítésben több laboratóriumi generáció, és a hibrideknek az eredeti populációkkal való visszakeresztezése is szerepel (Lynch & Walsh 1998; kidolgozott példáért lásd: Huttunen & Aspi 2003), egyszerűbb, egy generációs sémák is működnek (Wright 1978; Laugen et al. 2002). Mi egy ilyen egy generáció mélységű kísérletet végeztünk egy Balti-tengerből származó és egy attól több mint 900 km-re található tökéletesen izolált kis tavi populációt felhasználva. Három tulajdonságra koncentráltunk: testméret, ivarérés időzítése és táplálkozási aktivitás.

A megközelítés egyszerű: ha van két, az általunk vizsgált fenotípusos jellegben eltérő populációnk (A és B) akkor létrehozunk négy keresztezési vonalat (AA, BB, AB, BA; ahol az első betű az apa, a második pedig az anya populációját jelöli), majd összehasonlítjuk a *common garden* körülmények között felnevelt állatokat. Az interpretáció relatíve egyszerű (36. ábra):

1. Ha AA és BB eltér, a fenotípusos plaszticitás, mint egyetlen oka a természetben megfigyelt eltérésnek elvethető.
2. Ha AA és BB eltér, AB és BA viszont nem különbözik és nagyjából félúton található AA és BB között, akkor az additív genetikai komponens a meghatározó.
3. Ha AA és BB eltér, AB és BA viszont nem különbözik és vagy AA-hoz, vagy BB-hez van közel, akkor domináns genetikai hatások a döntőek.
4. Ha AA és BB eltér, AB és BA szintén különbözik, és AB a BB, BA pedig az AA populációhoz hasonló, akkor az anyai hatások alakítják a populációs eltéréseket.
5. Ha AA, BB, AB és BA laborgenerációi egyformák, akkor a természetben megfigyelt eltérés fenotipikus plaszticitás eredménye.

Persze ha több hatás párhuzamosan is jelentkezik, akkor az interpretáció bonyolódhat, és csak a második generáció (16 eltérő keresztezési vonal analízise, pl. Huttunen & Aspi 2003) interakciókat is tartalmazó vizsgálata adhat biztos választ.



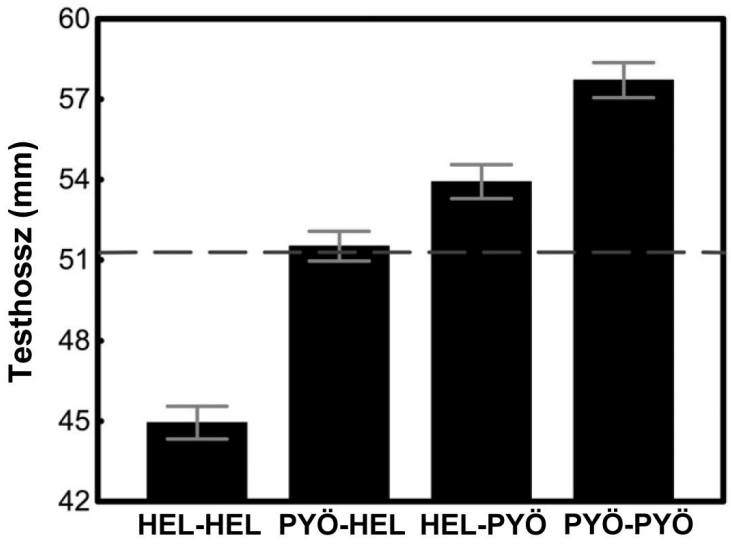
36. ábra. Sematikus reprezentációja a 4.3.1 pontban tárgyalt, a különböző tulajdonságokban megfigyelt populációs szétválás hátterét vizsgáló kvantitatív genetikai kísérletnek: két fenotipikusan eltérő (teli fekete *vs*. üres körök) populáció keresztezési kombinációinál laborban megfigyelhető fenotípusok (teli fekete és üres körök, valamint az átmeneti fenotípust jelképező szürke körök), és a belőlük következő kvantitív genetikai szcenáriók.

4.3.1.1 Testméret

Az első hibernáció utáni testméretet az apa és az anya populációja egyaránt befolyásolta, míg az interakció csak marginális szignifikanciát mutatott (Ab Ghani et al. 2012). Ez erős additív genetikai háttérre utal, és valóban, a tengeri és kis tavi

populációk mérete különbözött (37. ábra). Az interakció azonban gyenge domináns genetikai vagy anyai hatás jelenlétét is sejtette. A hibridek mind a tiszta vonalaktól, mind egymástól különböztek. A két tiszta vonal közötti középállapottal a tengeri anyától származó hibridek gyakorlatilag egybeestek, a kis tavi anyától származó hibridek viszont eltértek a középállapottól, és a kis tavi tiszta vonalhoz álltak közelebb (37. ábra). Ez a mintázat egy gyenge aszimmetrikus anyai hatásra utal.

A testméret ökológiai és evolúciós fontossága (Peters 1983; Roff 1992; Stearns 1992), illetve fajon belüli változatossága a természetben (Forsman 1991; Keogh et al. 2005; Lomolino 2005) egyaránt közismert. A populációs szétválás genetikai és környezeti meghatározottsága azonban már ritkábban felderített, holott a környezet által indukált fenotipikus plaszticitás egyedül is létrehozhat extrém méretkülönbségeket (Madsen & Shine 1993; Roff 2002). Ráadásul, az első laboratóriumi *common garden* generációk közötti eltérések is lehetnek még anyai vagy környezeti hatásoktól terheltek (Rossiter 1996; Lynch & Walsh 1998; Green 2008). Az eddigi vizsgálataink (4.1.3.1; Herczeg et al. 2009a, 2012) eredményei egyöntetűen a különböző kilenctüskés pikó populációk között megfigyelt méretbeli eltérések genetikai hátterét támogatták, de azt csak a jelen vizsgálat bizonyította, kimutatván a genetikai komponens additív voltát.



37. ábra. Testméretbeli különbségek (átlag ± standard hiba) tiszta és hibrid izolált kis tavi (PYÖ) és tengeri (HEL) kilenctüskés pikó populációk laborban felnevelt generációjánál 337 nappal a kikelés után. Az első rövidítés az apa, a második pedig az anya populációját jelöli. A vízszintes szaggatott vonal a tiszta populációk közötti intermedier állapotot jelöli. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

Az additív genetikai komponens mellett egy gyenge aszimmetrikus anyai hatást is megfigyeltünk. Az anyai hatások (általában a petébe juttatott anyagok mennyiségén keresztül) gyakran jelentősek az utódok méretének és növekedésének alakulásában halaknál, de a hatás főleg a korai időszakban jelentős (Green 2008). A mi esetünkben azonban az első hibernáció után, az első potenciális szaporodási

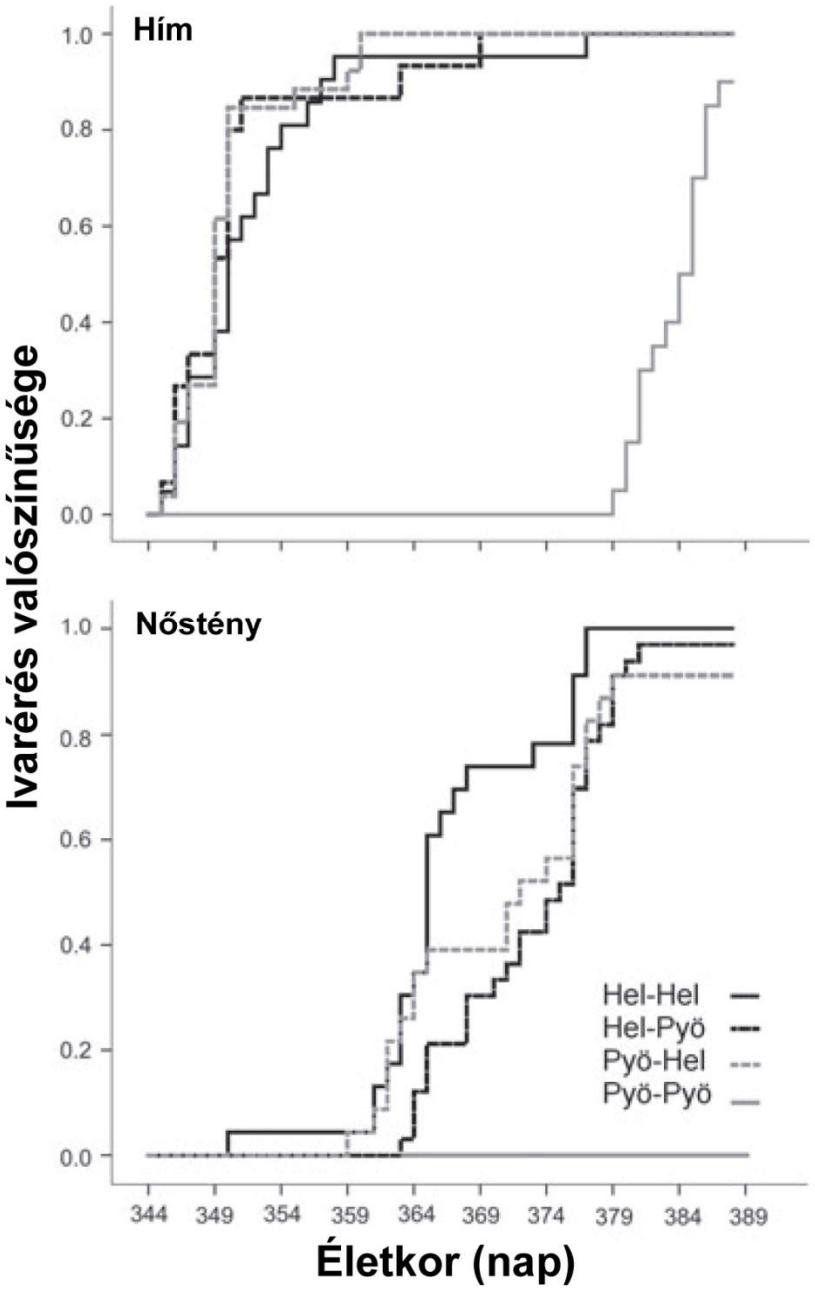
esemény környékén is kimutatható volt. A vizsgálatunk megmutatta, hogy a kis tavi óriás nőstények lényegesen nagyobb petéket termelnek, mint a normális méretű tengeri pikók (Ab Ghani et al. 2012) ami az anyai hatás mechanizmusát sejteti. Összességében az izolált kis tavakban megfigyelhető óriás testméret főként az additív genetikai háttérnek köszönhető, amire még ráerősít a kist tavi nőstények petébe való extra befektetésén keresztül manifesztálódó egyoldalú anyai hatás.

4.3.1.2 Ivarérés időzítése

Az ivarérés ideje tekintetében az eredményeink egyértelműek voltak: a vizsgált tulajdonságot az apa és az anya populációja, illetve ezek interakciója egyaránt befolyásolta (Ab Ghani et al. 2013). Ezek alapján a különbség nem magyarázható fenotipikus plaszticitással, viszont az egyszerű additív szcenárió sem kielégítő. A mintázatot jobban górcső alá véve az látjuk, hogy a tengeri és minkét hibrid vonal egyedei gyakorlatilag mind szaporodóképes állapotba kerültek a vizsgálat végére (nászszínezetet mutató hímek és petét termelő nőstények aránya az adott vonalon belül 96-100%), az izolált kis tavi pikóknál azonban csak az egyedek kisebb része vált ivaréretté (az egyedek 39%-a) (38. ábra). Ez a mintázat mintegy iskolapéldája a domináns genetikai hatások által kialakított populációs szétválásnak. Az ivarok is különböztek, a hímek előbb kerültek szaporodóképes állapotba, mint a nőstények, illetve a tiszta kis tavi vonalban kizárólag a hímek mutattak ivarérésre utaló jeleket (38. ábra).

Az ivarérés időzítése egy kiemelkedően fontos életmenet tulajdonság, ami feltehetően erős szelekciós nyomás alatt áll és ezért optimalizált a különböző környezetekben lévő populációkban (Stearns 1984; Reznick et al. 1990; Reznick & Ghalambor 2005). A szelekció általában az élettanilag lehetséges legkorábbi időpontban történő szaporodást részesíti előnyben, különösen predációs nyomás alatt álló populációkban (Stearns 1992; Hernaman & Munday 2005). Ugyanakkor a szaporodási siker és túlélés, illetve a jelenlegi és jövőbeli szaporodási siker közötti *trade-off* viszonyból eredően bizonyos körülmények között előnyös lehet a késleltetett ivarérés (Reznick et al. 1990; Roff 1992, 2002; Stearns 1992; Reznick & Ghalambor 2005). Az általunk tanulmányozott kilenctüskés pikó rendszerben a ragadozó halak hiányában evolválódó izolált kis tavi pikóknál, ahol az intraspecifikus versenyben (beleértve a szaporodást is) elért siker, és így a maximális méret jelenti a rátermettség növelésének eszközét, a késleltetett ivarérés előnyös stratégiának tűnt. Előzetes megfigyeléseink (Herczeg et al. 2009a) támogatták ezt a hipotézist, a jelen vizsgálat pedig bizonyította azt.

A speciális keresztezési sémánk analízise a populációs szétválás domináns genetikai hátterét bizonyította. A korai ivarérést találtuk a domináns tulajdonság-állapotnak. Mivel a tengeri pikók tekinthetőek az ősi, a kis tavi pikók pedig a leszármazott formának, ez az eredmény azt mutatja, hogy a kis tavakat meghódító pikókra igen erős szelekció hathatott a késleltetett ivarérés érdekében, hiszen egy recesszív tulajdonság elterjedését okozta.



38. ábra. Az ivarérés valószínűségének különbségei tiszta és hibrid izolált kis tavi (PYÖ) és tengeri (HEL) kilenctüskés pikó populációk laborban felnevelt generációjánál nyolc héttel a mesterséges hibernáció után. Az első rövidítés az apa, a második pedig az anya populációját jelöli. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

4.3.1.3 Táplálkozási aktivitás

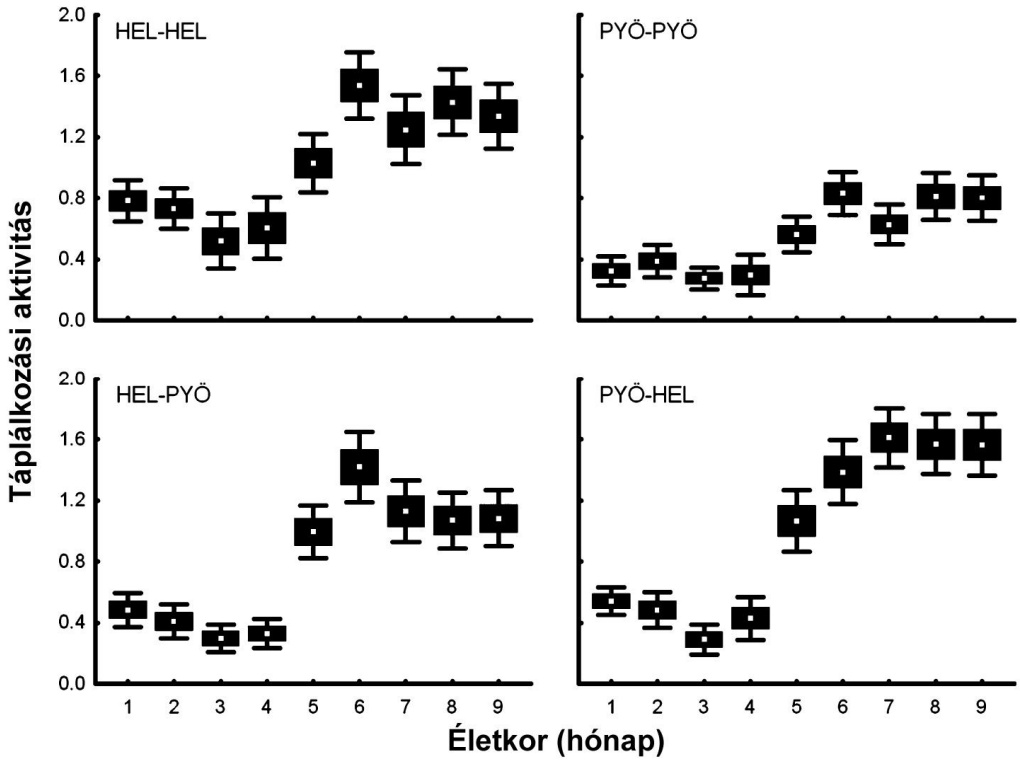
A táplálkozási aktivitás változatosságát kilenc hónapon keresztül követtük havi bontásban, és így a kvantitatív genetikai analízisünk egy egyedfejlődési dimenziót is kapott (Herczeg et al. 2013). Az egyedek között mind a viselkedési típus, mind az ontogenetikus viselkedési plaszticitás szignifikánsan eltért, tehát az állati személyiségek (*animal personality*) és azok plaszticitása egyaránt bizonyítottnak

tekinthetőek (Dingemanse et al. 2010). A mintázatokat egy hármas interakció, az anya és az apa populációjának és az egyedfejlődési stádiumnak az interakciója határozta meg. Tehát az eltéréseknek van genetikai háttere, az egyszerű additív genetikai hipotézis nem kielégítő, és a kvantitatív genetikai meghatározottság függ az egyedfejlődési stádiumtól. Bár a teljes vizsgált egyedfejlődési periódust tekintve egy szisztematikus csökkenést figyelhetünk meg, a viselkedési mintázatok tisztán elkülöníthetőek voltak egy korai és egy késői egyedfejlődési szakaszra (39. ábra). A korai szakaszban a tiszta tengeri vonal egyértelműen alacsonyabb táplálkozási aktivitást mutatott, mint a különböző hibridek vagy a tiszta kis tavi vonal, míg ez utóbbi három nem tért el egymástól (39. ábra). Tehát ebben a szakaszban domináns genetikai hatások állnak a populációs szétválás mögött, ahol a kis tavi populációban található(ak) a domináns allél(ok). A késői szakasz egy kissé összetettebb képet mutatott. Itt a tiszta kis tavi vonal egyértelműen magasabb táplálkozási aktivitást mutatott, mint a hibridek vagy a tengeri populáció, ámde ez utóbbi három nem volt homogén (39. ábra). A tengeri anyáktól származó hibridek és a tiszta tengeri vonal nem tért el, és a legalacsonyabb táplálkozási aktivitást mutatta, a kis tavi anyáktól származó hibridek viszont a tiszta tengeri és kis tavi vonalak között nagyjából félúton helyezkedtek el (39. ábra). Ez a mintázat alapvetően domináns genetikai hatásokra utal, domináns tengeri allél(okk)al, de egy aszimmetrikus anyai hatás is megfigyelhető.

A konzisztens egyedi különbségek a viselkedési típusban és viselkedési plaszticitásban érdekesek az állati személyiség kutatásában (lásd pl. Gosling 2001; Sih et al. 2004a,b; Dingemanse et al. 2010; Wolf & Weissing 2010), de a jelen dolgozat mondanivalója szempontjából nem fontosak, ezért ezek tárgyalására nem térek ki.

Az egyedfejlődés alatti táplálkozási aktivitás csökkenés általános trendként jelentkezett, bár mérteke eltért az egyedek és a keresztezési vonalak között. Az aktivitás általános költsége a ragadozókkal való találkozás növekvő valószínűsége (Lima & Dill 1990). Fiatal egyedek esetében jobban megéri kockáztatni, hiszen az ivarérett méret eléréséhez sok táplálékra van szükség, és a nagyobb testméret a méret-limitált ragadozók jelentette veszély megszűnését is jelenti (Abrams & Rowe 1996; Urban 2008). Később azonban a drasztikus méretváltozás lehetősége csökken, és így a magas aktivitás relatív költsége megnő, és az ivarérett méret elérésekor már a túlélés a kritikus rátermettség komponens. Dacára azonban az egyedfejlődés alatti általános trendnek, a különböző keresztezési vonalak markánsan eltértek. A legnagyobb eltérés a tiszta vonalak között volt, a kis tavi populáció pikói minden egyedfejlődési stádiumban magasabb aktivitást mutattak, mint a tengeri fajtársaik és az aktivitás csökkenése is a tengeri pikóknál volt kifejezettebb. Ez tökéletesen egybecseng a két populáció között megfigyelt növekedési stratégiabeli eltéréssel: a kis tavi pikók abszolút mértékben gyorsabban fejlődnek, mint a tengeriek, de a növekedési periódusuk sokkal hosszabb ideig tart (4.1.3.1; Herczeg et al. 2012). A hibridek vizsgálatával komplex genetikai háttér került napvilágra (Wright 1978;

Lynch & Walsh 1998; Laugen et al. 2002): a fiatal halaknál a kis tavi populáció hordozta magas táplálkozási aktivitást kódoló allélok, míg az ivarérett méret közelébe kerülő halaknál a tengeri populáció hordozta alacsony táplálkozási aktivitást kódoló allélok voltak dominánsak. Mivel a tengeri típus tekinthető ősinek, a kis tavi élettér meghódításával járó genetikai adaptáció egy domináns karakter (korai táplálkozási aktivitás) evolúcióját és egy recesszív karakter (késői táplálkozási aktivitás) fixációját is magába foglalta.



39. ábra. Táplálkozási aktivitásbeli különbségek (átlag ± standard hiba + 95% konfidencia intervallum) tiszta és hibrid izolált kis tavi (PYÖ) és tengeri (HEL) kilenctüskés pikó populációk laborban felnevelt generációjánál kilenc hónapon keresztül. Az alacsony értékek magas aktivitást jelölnek. Az első rövidítés az apa, a második pedig az anya populációját jelöli. A populációs kódok megtalálhatóak az 1. táblázatban és a 3. ábrán.

A domináns genetikai hatások mellett a testméret analízisekor (4.3.1.1; Ab Ghani et al. 2012;) találthoz hasonlító aszimmetrikus anyai hatás is jelen volt; a kis tavi anyák hibrid utódai magasabb késői táplálkozási aktivitást mutattak, mint a tengeri anyák hibrid utódai. Az anyai hatások fontossága a természetben megfigyelhető fenotípusos változatosság kialakításában széles körben ismert (Kaplan 1998; Mousseau & Fox 1998). Környezeti vagy anyai források allokációján keresztül hatnak (Mousseau & Fox 1998; Rossiter 1998; Green 2008), és a hatásuk több generáción keresztül is megfigyelhető (Lacey 1998; Rossiter 1998; Green 2008). A mi esetünkben az anyák (a genetikai anyagukat leszámítva) kizárólag a petéik összetételén keresztül vagy örökölhető nem-genetikai hatásokon keresztül

befolyásolhatták az utódaikat. A pontos mechanizmus a mi adataink alapján nem volt meghatározható. Mindenestre a kis tavi kilenctüskés pikóknál minden megfigyelt hatás (genetikai és anyai) a magasabb táplálkozási aktivitást és a nagyobb testméret elérését segítette.

4.3.2 Szelekció alatt álló genom-régiók

Az evolúcióbiológia egyik ultimális célkitűzése a természetben megfigyelt fenotípusos változatosságért felelős gének feltérképezése. Ennek két fő módja van. Amennyiben az irodalomból már ismert bizonyos gének szerepe egyes fajoknál, ugyanezen gének szerepét tesztelhetjük az általunk vizsgált taxonnál is. Az ilyen megközelítést ahol általában csak néhány, előre kiválasztott gén szerepének tesztelésére szorítkozunk, kandidáns gén analízisnek (*candidate gene analysis*; Palopoli & Patel 1996) nevezzük. Bár a teljes genomot vizsgáló módszerekhez képest ez a megközelítés egyszerű és olcsó, az eredmények kiszámíthatatlanok (a módszert gyakran hasonlítják az orosz ruletthez), és még pozitív eredmények birtokában sem ismerhetjük a vizsgált tulajdonság teljes genetikai hátterét. Ezért a genomikai módszerek tárházának drasztikus kibővülése nyomán napjainkban a módszer háttérbe szorulni látszik az újabb, teljes genomot szűrő megközelítések mellett. Ezek közül a leggyakoribbak a már korábban tárgyalt (1.3) *genome scan* vagy *hitchhiking mapping* (Schlötterer 2003; Storz 2005; Vasemägi & Primmer 2005) és a *Quantitative Trait Locus* [*QTL*] *mapping* (Erickson *et al*. 2004; Slate 2005). Vizsgálatainkban mindkét módszert alkalmaztuk a kilenctüskés pikó természetes szelekció alatt álló genomrégióinak feltérképezéséhez.

4.3.2.1 *Genome scan*

A természetes szelekció genomikus nyomainak azonosítása történhet a fenotípus figyelembe vétele nélkül, pusztán a genom szakaszainak neutralitás-tesztjén keresztül a *genome scan* vagy *hitchhiking mapping* módszerrel (Schlötterer 2003; Storz 2005). A kandidáns gén analízisnél megfigyelt 'orosz rulett' effektus elkerülése érdekében a lehető legnagyobb számú neutrális markerre van szükség a genom lehető legjobb lefedettségéhez (Vasemägi & Primmer 2005). Ez persze komoly logisztikai és módszertani kihívásokat, illetve idő- és költségbeli kényszereket jelent. Jelen vizsgálatunk (Bruneaux et al. 2013) egyik célkitűzése egy új, költség és időhatékony, nagy lefedettséget generáló módszer (*paired-end double restriction-site-associated DNA*) tesztelése volt. A módszertani részletek a dolgozatom szempontjából irrelevánsak, ezért a részletes ismertetésükre nem térek ki. Az idevágó másik célkitűzésünk a szelekció nyomainak kiszűrése volt a kilenctüskés pikó genomban az új módszer segítségével. Két tengeri, két nagy tavi és négy izolált kis tavi populáció populációnként összesített DNS-ét használtuk fel, illetve referenciaként bevontunk egy háromtüskés pikó populációt is (Bruneaux et al. 2013).

Az izolált kis tavakban megfigyelt alacsony genetikai diverzitás nem tette lehetővé a tervezett tenger – nagy tó – kis tó összehasonlítást, csak tengeri – édesvízi

összevetésre volt lehetőségünk. Ezért az eredeti célkitűzésünk, azaz a kis tavi adaptációk genomikai feltérképezése, nem valósulhatott meg. A különböző sókoncentrációkhoz való alkalmazkodás genetikai részleteiről a kilenctüskés pikónál egy 48 neutrális és 63 *a priori* kiválasztott génhez fizikailag kapcsolt mikroszatellita marker alapú *genome scan* alapján már vannak eredmények: a Növekedési Homron Receptor 2 (*Growth hormone receptor 2*) és a DEAD box Polipeptid 56 (*DEAD box polypeptide 56*) gének habitat alapú elkülönülése a különböző sókoncentrációkhoz való adaptációban játszott szerepükre utal (Shikano et al. 2010b). Az általunk használt új módszerrel a 111 mikroszatellita markernél nagyobb lefedettséget eredményező 6834 SNP-t (egypontos nukleotid polimorfizmus, *single nucleotide polymorphism*) vontunk analízisbe, és kilenc genomrégiót (nyolc kromoszómán) találtunk a neutrálisnál magasabb illetve nyolc genomrégiót (nyolc kromoszómán) a neutrálisnál alacsonyabb divergenciával (Bruneaux et al. 2013). A megnövekedett divergencia a szétválasztó, a csökkent divergencia pedig a stabilizáló természetes szelekció nyomaként értelmezhető. A szétválasztó szelekció alatti régiókban főleg a vese fejlődése és funkciója, az immunfunkciók, és a Mitogén-Aktivált Protein (MAP) kináz (*Mitogen-activated Protein Kinase*) útvonalak szempontjából fontos gének szerepeltek nagy számban, míg stabilizáló szelekció alatt a hemosztázisért, lipid metabolizmusért és szignalizációért, valamint az energiaraktárak metabolizmusáért felelős génekben gazdag régiók voltak. Noha ezek a mintázatok intuitíven logikusak (pl. az ozmoreguláció értelemszerűen fontos az eltérő sókoncentrációkhoz való alkalmazkodásban), meglepő módon a jelen vizsgálatban kimutatott szelekció alatt álló régiók nem egyeztek meg a háromtüskés pikókon folytatott hasonló vizsgálatokban kimutatott régiókkal (Hohenlohe et al. 2010; DeFaveri et al. 2011), sőt, egy eltérő kilenctüskés pikó populációkra alapozott vizsgálatban talált régiókkal (Shikano et al. 2010b) sem. Az inkongruenciának számos oka lehet, de általánosságban kijelenthető, hogy a fenotípus szintjén sok esetben megfigyelhető párhuzamos vagy konvergens evolúciós mintázatok genetikai háttere közelrokon fajok között, de akár fajon belül is eltérhet.

4.3.2.2 *QTL mapping*

Mint azt az előző fejezetben (4.3.2.1) láttuk, az izolált kis tavi kilenctüskés pikóknál megfigyelt alacsony genetikai diverzitás (4.1.1; Shikano et al. 2010a, Bruneaux et al. 2013) nem teszi lehetővé a speciális kis tavi adaptációk genomikai vizsgálatát a *genome scan* módszerével. A *QTL mapping* laboratóriumi szaporításon alapuló módszerénél azonban mi magunk hozunk létre mesterséges (hibrid) populációt, és ezért az alacsony genetikai diverzitás általában nem lehet probléma. Továbbá ez a módszer alkalmas előre kiválasztott fenotípusos tulajdonságok genetikai hátterének célzott feltárására, és ezért sok szempontból előnyösebb, mint a *genome scan*.

A 4.1 részben összefoglaltam azokat a vizsgált tulajdonságokat, amelyekben az izolált kis tavi kilenctüskés pikók szisztematikusan eltérnek a tengeri vagy nagy tavi fajtársaiktól. A 4.2 pontban tárgyaltam a legrelevánsabb tulajdonságok fenotípusos

plaszticitását, jelen részben pedig kísérletet teszek a releváns tulajdonságokban megfigyelt populációs szétválás genomikai hátterének azonosítására.

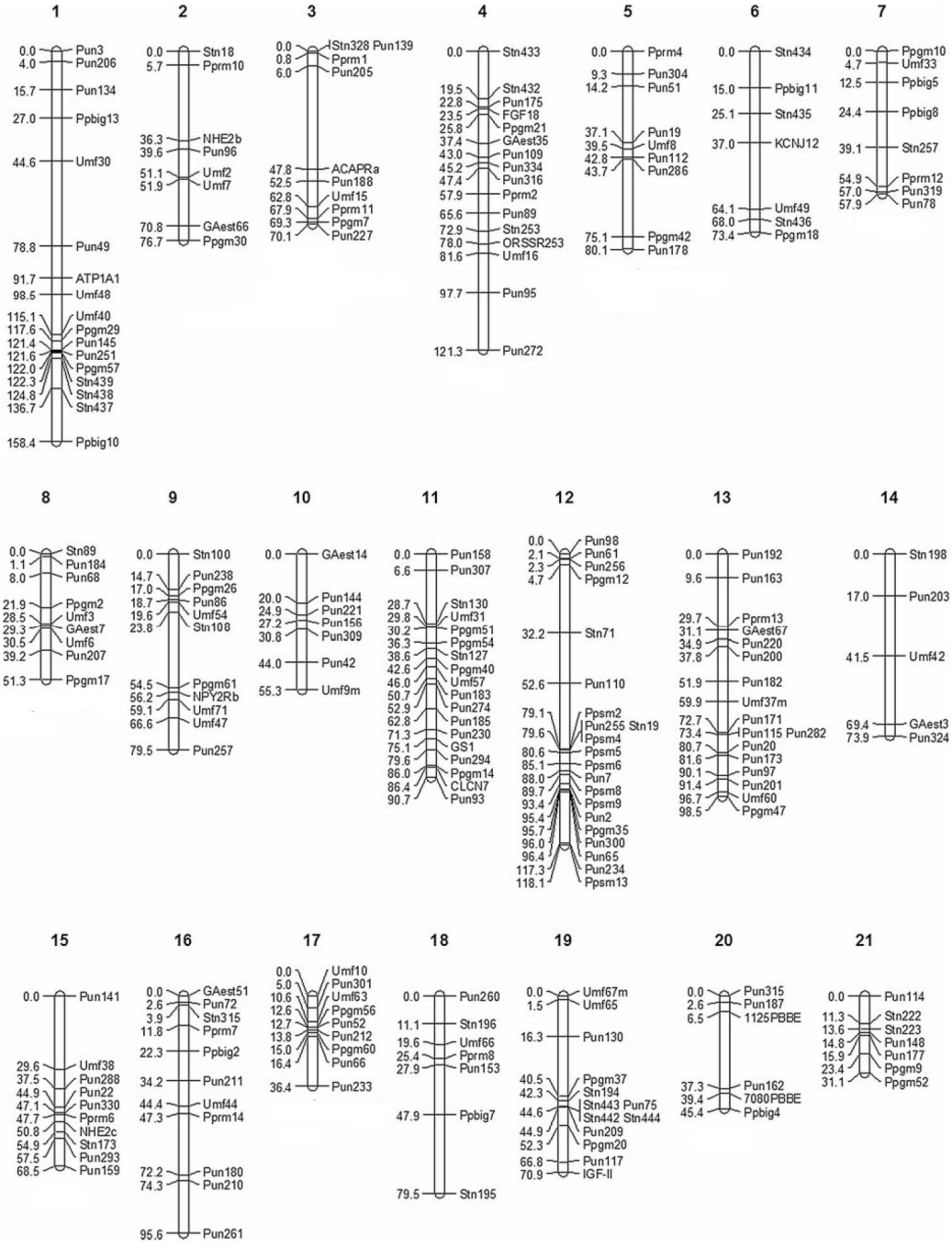
A *QTL mapping* módszere röviden: egy ismert pedigré mentén keresünk asszociációt a fenotípusos és genetikai változatosság között (Erickson *et al*. 2004; Slate 2005). A konkrét megközelítések száma nagy, a természetben követett sok generációtól a tervezett laboratóriumi keresztezésekig. Mi a vizsgált faj karakterisztikáiból adódóan a második módszert, és azon belül is az *inbred line cross design*-t (Lynch & Walsh 1998) alkalmaztuk. Először kereszteztünk egy hím és egy nőstény egyedet az ismert legszélsőségesebb populációk között (izolált kis tó: Rytilampi, Finnország; tenger: Balti-tenger, Finnország; „RYT" és „HEL" a 3. ábrán és 1. táblázatban). Az ebből a keresztezésből származó F1 családot felneveltük, majd mesterséges hibernáció után egy kiválasztott párt hétszer egymás után kereszteztünk (az egyedek „természetes" módon párosodhattak). Az F2 generációt szintén felneveltük, a vizsgált tulajdonságaikat lemértük, majd a genotípusukat is meghatároztuk. Összességében 283 második generációs halat vizsgáltunk (56, 44, 38, 37, 40, 31 és 27 egyed a hét családból). A genetikai adatbázist 226 mikroszatellita marker alapján képeztük (235-ből 226 bizonyult informatívnak). Ezek közül 46 marker előre kiválasztott kandidáns génekhez (ismert biológiai funkcióval) kapcsolt volt, így a random markerek által reprezentált random genomikai minta mellett ezeket a géneket tudatosan céloztuk. Tíz marker növekedéshez (Shikano et al. 2010a), 13 viselkedéshez (Laine et al. 2012b), a maradék 23 pedig általános élettani funkciókhoz (Shikano et al. 2010a; Shimada et al. 2011) köthető génekhez volt tervezve.

Első lépésként, a 283 második generációs pikó genotípusa mellett a szülők és nagyszülők genotípusainak bevonásával és a publikált háromtüskés pikó (Ensembl, adatbázis v. 66.1) és észak-amerikai kilenctüskés pikó genomtérképek (Shapiro et al. 2009) felhasználásával rekonstruáltuk az általunk vizsgált kilenctüskés pikó klád genomtérképét (40. ábra). Ezen a térképen a 226 mikroszatellita marker fizikai helye ismert és így az adott markerekkel kovariáló fenotípusos tulajdonságokért felelős gének fizikai helye is becsülhető. A kapcsolódási csoportok (*linkage group*) nagy valószínűséggel megfeleltethetőek a kromoszómáknak (Ocalewicz et al. 2008), ezért mostantól kromoszómaként említem őket. Az alábbiakban a vizsgált fenotípusos tulajdonságok sorrendjében ismertetem az eredményeinket.

4.3.2.2.1 A hasi tüske és a mell-öv redukciójának genetikai háttere

A háromtüskés pikó már évtizedek óta, a kilenctüskés pikó pedig napjainkra már szintén modellfajnak számít a párhuzamos és konvergens evolúció vizsgálatában (Bell et al. 1994; Merilä 2013). A fajon belüli dokumentált nagy fenotípusos változatosság habitat-függésén, és a különböző fenotípusok ismételt, egymástól független evolúcióján kívül a modellé válás fontos feltétele a háromtüskés pikóról rendelkezésre álló részletes genomikai információ (Colosimo et al. 2004, 2005; Cresko et al. 2004; Shapiro et al 2004), amit könnyen lehetett a kilenctüskés pikóra is

adaptálni (Shapiro et al. 2009; Shikano et al. 2010a). Így a két fajnál ismert fenotípusos evolúció közvetlen genetikai háttere felderíthető, és ez által az evolúcióbiológia egyik ultimális célja – a természetben megfigyelt adaptív fenotípusos változatosság genetikai hátterének leírása – megvalósítható.



40. ábra. A kilenctüskés pikó genomikai kapcsolódási térképe. 21 kromoszómának megfeleltethető kapcsolódási csoportot találtunk. Jobb oldalt a felhasznált mikroszatellita markerek (N=226) nevei, a bal oldalon pedig a genetikai távolságok (centimorganban) vannak feltüntetve.

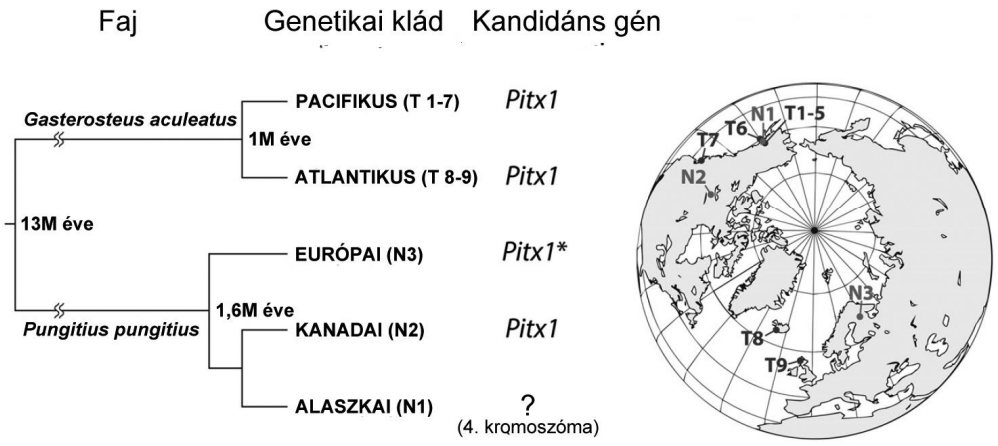
A két pikófaj fenotípusos változatosságát illetően a morfológiai tulajdonságok kapták a legnagyobb hangsúlyt. Az egyik kulcs-tulajdonság a hasi tüske és a mell-öv fejlettsége. Ezen képletek a méret-limitált ragadozók elleni védekezés frontvonalát jelentik. A pikóknál gyakran megfigyelt redukció, extrém esetekben hiány, általában a ragadozókkal, illetve a víz oldott iontartalmával függ össze (Giles 1983; Bell et al. 1993; Marchinko & Schluter 2007; Marchinko 2009; Leinonen et al. 2011). A mell-öv és a hasi tüske redukciójának genetikai háttere a háromtüskés pikónál már ismert, a *pituitary homeobox transcription factor 1* (*Pitx1*, magyar neve számomra ismeretlen) gén felelős a változatosság oroszlánrészéért (Shapiro et al. 2004; Coyle et al. 2007; Chan et al. 2010). A kilenctüskés pikóknál a helyzet nem ilyen egyszerű. Egy, a három- és (kanadai) kilenctüskés pikók hibridjeinek fenotípusos analízisén alapuló vizsgálat az utóbbi fajnál is a *Pitx1* gén szerepét sejtette (Shapiro et al. 2006). Ugyanakkor egy monomorfikus redukált kanadai és polimorfikus redukált alaszkai populáció keresztezésén alapuló *QTL mapping* analízis már *Pitx1*-től eltérő genomikai régió szerepét mutatta ki (Shapiro et al. 2009). Jelen vizsgálatunkban (Shikano et al. 2013) a kilenctüskés pikó európai kládjával végeztünk *QTL mapping* kísérletet a mellkasi védelmi struktúrák genetikai háttere feltárásának érdekében.

	HEL/HEL (N=70)	HEL/RYT1 (N=60)	HEL/RYT2 (N=73)	RYT1/RYT2 (N=68)
Bal hasi tüske	3,62 ± 0,09	3,22 ± 0,11	3,41 ± 0,07	0,47 ± 0,13
Jobb hasi tüske	3,49 ± 0,09	3,23 ± 0,11	3,32 ± 0,07	0,35 ± 0,12
Mell-öv bal oldala	7,98 ± 0,13	7,44 ± 0,16	7,41 ± 0,09	3,90 ± 0,18
Mell öv jobb oldala	7,61 ± 0,13	7,14 ± 0,16	7,11 ± 0,08	4,37 ± 0,17

3. táblázat. A hasi tüske és mell-öv hossza (mm; átlag ± standard hiba) a különböző *Pun319* genotípusokban. Egy tengeri (HEL) és két izolált kis tavi (RYT1, RYT2) allélt találtunk.

A kis tavi (Rytilampi) populációban az összes ismert egyednél hiányzik a hasi tüske, a mell-öv pedig erősen redukált, ellentétben a kifejezett tüskével és mell-övvel bíró balti-tengeri populáció egyedeivel (4.1.2; Herczeg et al. 2010a). A jelen vizsgálatban az F1 generáció minden tagjának kifejezett tüskéje volt, az F2 generációban pedig 218 egyednek volt hasi tüskéje, 65-nek pedig nem. Ez a mintázat megfelel az egyszerű egygénes mendeli öröklésmenetnek. Mind a jobb és bal hasi tüske, mind a mell-öv jobb és bal oldali egységének hossza erős összefüggést mutatott a 7. kromoszómán található *Pitx1* génben lévő *Pun319* mikroszatellita marker változatosságával (a változatosság 58-69%-át fedték le). A Rytilampi allélokat hordozó halak hasi tüskéi és a mell-öve redukált volt a többi allélkombinációkhoz képest. Kisebb mértékben ugyan, de a tisztán balti-tengeri allélokat hordozó halak is eltértek a heterozigótáktól; itt voltak a legkifejezettebbek a vizsgált tulajdonságok (3. táblázat). A genom más helyével nem találtunk összefüggést, beleértve a Shapiro és munkatársai (2009) által, az észak-amerikai kládban kimutatott pozíciót is.

Összességében az eredményeink egybecsengenek a háromtüskés pikónál találtakkal (Cresko et al. 2004; Shapiro et al. 2004; Coyle et al. 2007): a mellkasi védelmi struktúra redukciója a *Pitx1* gén által, recesszív mendeli módon meghatározott az általunk vizsgált európai kládnál, az észak-amerikai kláddal ellentétben (41. ábra). Ez két érdekes és első hallásra ellentmondó jelenségre hívja fel a figyelmet. Először, a két, egymástól több mint 10 millió éve (Bell et al. 2009) elvált pikófaj esetében is működhetett ugyanaz a genetikai mechanizmus az azonos fenotípusos adaptáció mögött. Másodszor, a kevesebb, mint 1,6 millió éve elvált észak-amerikai és európai kilenctüskés pikó kládok (Teacher et al. 2011) más-más genetikai megoldással jutottak el ugyanahhoz a fenotípusos adaptációhoz: legalább egy észak-amerikai populációban a *Pitx1*-től független genomikai régió felel a mellkasi védelmi struktúra redukciójáért. A háromtüskés pikó esetében a hasi tüske és mell-öv mellett az oldalsó pajzsok redukcióját is ugyanazok a genetikai mechanizmusok irányítják (hasi tüske és mell-öv: *Pitx1* gén, oldalsó pajzsok: *ectodysplasin* gén) az atlanti és a pacifikus kládokban egyaránt (Cresko et al. 2004; Shapiro et al. 2004; Colosimo et al. 2005; Coyle et al. 2007). A kilenctüskés pikó esetében megfigyelt sokkal nagyobb számú klád (Takahashi & Goto 2001; Aldenhoven et al. 2010; Shikano et al. 2010c; Teacher et al. 2011) megnöveli a valószínűségét az alternatív genetikai mechanizmusoknak. Ezt az elképzelést a mellkasi védelmi struktúra redukciója mögött álló eltérő genetikai mechanizmusok alátámasztják, de nyilván több fajon belüli és fajok közötti genomikai összehasonításra lesz szükség az általánosításhoz.



41. ábra. Filogenetikai kapcsolatok és a mellkasi védelmi struktúra redukciójáért felelős kandidáns gének a három- és kilenctüskés pikó kládjainál. A törzsfa genetikai adatokon (Haglund et al. 1992; Ortí et al. 1994; Aldenhoven et al. 2010; Teacher et al. 2011) és fossziliák analízisén (Bell et al. 2009) alapszik. A kandidáns géneket korábbi (Cole et al. 2003; Cresko et al. 2004; Shapiro et al 2004, 2006, 2009; Coyle et al. 2007; Chan et al. 2010) és a jelenlegi (*) vizsgálatokban határozták meg.

A fenotipikus változatosságért felelős „fő" gének mellett gyakran megfigyelhetőek „módosító" gének (*modifier genes*) is, tipikusan sokkal kisebb hatással a vizsgált fenotípusos tulajdonságra. Négy módosító gént valószínűsítettek a háromtüskés pikó esetében (Shapiro et al. 2004) és egyet az észak-amerikai

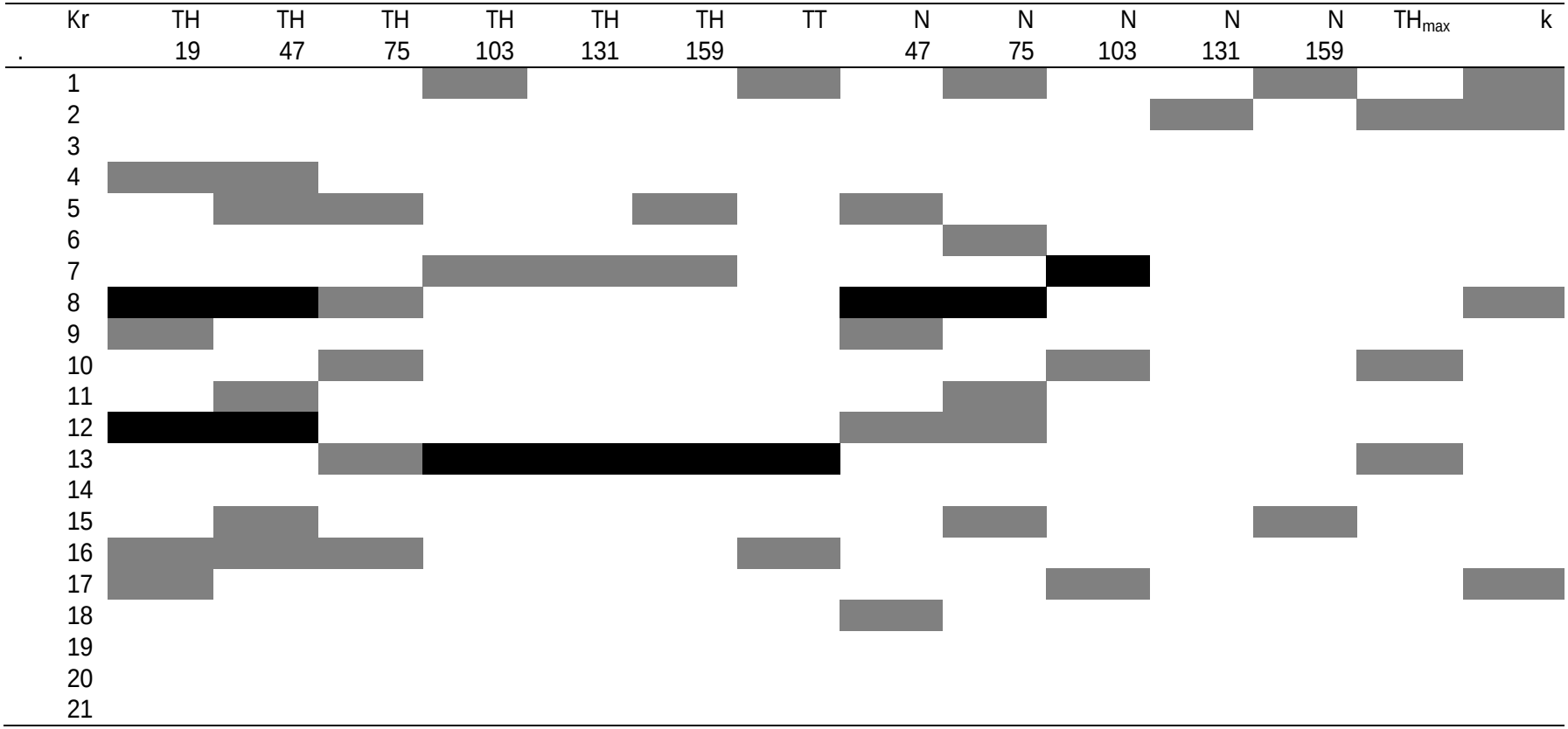
kilenctüskés pikóknál (Shapiro et al. 2009). A mi vizsgálatunkban nem találtunk bizonyítékot módosító génekre. Valószínűleg a relatíve kis hatású módosító gének száma és genombeli helye nagy változatosságot mutat mind fajok között, mind fajon belül, habár megjegyzendő, hogy a kis hatással bíró gének azonosításában sokkal nagyobb a hiba esélye, mint a fenotipikus variancia nagy részéért felelős fő gének esetében.

4.3.2.2.2 A testméret és a növekedési ráta genetikai háttere

Az előző fejezetben tárgyalt pikó-specifikus tulajdonságok mellett több, általános kvantitatív tulajdonságban is feltártunk populációs divergenciát a dolgozatomban tárgyalt kilenctüskés pikó rendszerben (4.1). Ezek között is kiemelkedően nagy jelentőségű a testméret és a növekedési ráta (Peters 1983, Roff 1992; Stearns 1992; Arendt 1997). Mindkét tulajdonság széles körben kutatott, és nem meglepő, hogy genetikailag meghatározott populációs szétválást sok esetben publikáltak (Blanckenhorn 2000; Dmitriew 2011). Ezen tulajdonságok heritabilitása tipikusan közepes-magas (Lynch & Walsh 1998; Gjedrem 2000). Noha az ilyen jellegű ismeretek kiválóan alkalmasak sok evolúciós következtetés levonására, a pontos mechanizmusok megértése nem lehetséges a genetikai háttér ismerete nélkül (Mackay et al. 2009; Hill & Kirkpatrick 2011).

Mind a testméret, mind a növekedési ráta tekintetében ismétlődő független evolúciót figyeltünk meg az izolált kis tavakban, ahol gyakran óriás méretű pikók élnek, az óriás méret elérését célzó növekedési és ivarérési stratégiát mutatva (Herczeg et al. 2009a; 2012; Ab Ghani et al. 2013; Aikio et al. 2013). Bár a testméretben megfigyelt változatosság tipikusan sok gén által szabályozott jelenség (Anderson & Georges 2004; Rocha et al. 2004), a növekedési rátákban megfigyelt fő különbség (a tengeri pikók növekedése megtorpan egy bizonyos méret elérésekor, a kis tavi pikók növekedése viszont tovább folytatódik) alapján elképzelhető egy egyszerű genetikai mechanizmus is a növekedés „kikapcsolása” mögött. Az itt tárgyalt vizsgálatban (Laine et al. 2013) a kilenctüskés pikók európai kládjának *QTL mapping* analízisét végeztük el a testméretbeli és növekedési rátabeli különbségek genomikai hátterét feltérképezendő.

A testméretet és növekedési rátát több változóval jellemeztük. Lemértük a halak hosszát 19, 47, 75, 103, 131 és 159 napos korukban. Ezeket a változókat felhasználtuk külön-külön is, és belőlük kalkuláltuk a von Bertalanffy növekedési paramétereket is (4.1.3.1; von Bertelanffy 1938). Kalkuláltunk még stádiumonkénti növekedést is az egymást követő hosszadatok különbségeként. Végezetül, analizáltuk a kísérlet végén (187 napos korban) mért testtömeget. A különböző (korreláló) változóink számos genomrégióval voltak kapcsolatban (megmagyarázott változatosság 3 és 12% között; 4. táblázat). Általánosan elmondható volt, hogy



4. táblázat. A különböző, testméretet és növekedési stratégiát leíró változók és kandidáns genomikai régiók kapcsolata. Kr = kromoszóma, TH = testhossz, TT = testtömeg, N = növekedés, THmax = a von Bertalanffy modell által becsült maximális testhossz, k = a von Bertalanffy modell által becsült növekedési konstans. Testhosszt 19, 47, 75, 103, 131 és 159 napos korban mértünk, növekedést 47, 75, 103, 131 és 159 napos korban becsültünk, a von Bertalanfy görbéket az egyedenként hatszor felvett hosszadatokra illesztettük, testtömeget a kísérlet végén, 187 napos korban mértünk. A szürke szín kromoszóma-szintű, a fekete szín pedig genom-szintű szignifikáns kapcsolatot jelez.

a korai stádiumban mért változatosság mögött több genomrégió állt, mint a későbbi stádiumban tapasztalt változatosság mögött (4. táblázat). Ráadásul a két stádium nagyrészt más-más genomrégiókkal mutatott kapcsolatot. Két feltételezett kromoszómán (8. – korai szakasz és 13. – késői szakasz) is erős, sok változót érintő hatást találtunk. Az adott szakaszt jellemző markerek allél eloszlásai egységes képet mutattak: a kis tavi allélekre homozigóta egyedek nagyobbak voltak, mint a heterozigóta, vagy tengeri allélekre homozigóta egyedek (5. táblázat). Nem találtunk a növekedési stratégiákra speciálisan (mérettől függetlenül) ható genomrégiót. Bár a kapcsolatot mutató genomrégiók elég szélesek voltak, egy növekedési, két viselkedési és négy élettani génnel való kapcsolatot tudtunk valószínűsíteni (részleteket lásd Laine et al. 2013 a Függelékben, 9).

Ppgm2; 8. kromoszóma	TH1	TH2	TH3
MM	11,70 ± 0,68	22,82 ± 2,14	31,63 ± 2,38
MP	11,98 ± 0,77	23,83 ± 1,87	32,30 ± 2,42
PP	12,11 ± 0,68	24,64 ± 1,93	32,90 ± 3,22
Pun173; 13. kromoszóma	TH4	TH5	TH6
Mm	40,53 ± 3,24	45,54 ± 3,60	47,86 ± 0,43
MP	41,48 ± 3,55	46,19 ± 4,11	48,49 ± 0,44
mP	41,69 ± 2,73	46,59 ± 3,21	48,95 ± 0,38
PP	42,95 ±3,36	48,38 ± 3,91	51,29 ± 0,40

5. táblázat. A testhossz (mm; átlag ± standard hiba) a különböző életkorokban, és *Ppgm2* és *Pun173* genotípusokban. *Ppgm2*: egy tengeri (M) és egy kis tavi (P) allélt találtunk. *Pun173*: két tengeri (M, m) és egy kis tavi (P) allélt találtunk. TH1 = 19 napos, TH2 = 47 napos, TH3 = 75 napos, TH4 = 103 napos, TH5 = 131 napos és TH6 = 159 napos kor.

Az eredményeink egyértelműen elvetették a méretbeli és növekedési stratégiabeli szétválás mögötti egyszerű genetikai háttér (be/kikapcsolás) lehetőségét (pl. Hosoya et al. 2012), és a sokkal gyakoribb „sok gén kis hatással" modellt (pl. Anderson & Georges 2004; Rocha et al. 2004) támogatták. Azt, hogy más gének befolyásolják a méretet/növekedést az eltérő egyedfejlődési stádiumokban megfigyelték egereknél (Cheverud et al. 1996; Rocha et al. 2004; Allan et al. 2005) és csirkéknél is (Carlborg et al. 2003; Podisi et al. 2013). Az additív hatások relatív többsége és a kis tavi allélok testméretre kifejtett pozitív hatása egybecseng a korábbi egyszerű kvantitatív genetikai kísérletünk (4.3.1.1; Ab Ghani et al. 2012) eredményeivel. A tény, miszerint a méretet/növekedést kevesebb gén befolyásolta az egyedfejlődés későbbi szakaszában, felveti a lehetőségét a környezeti faktorok megnövekedett szerepének (cf. Podisi et al. 2013). Ez a felvetés intuitíve is logikusnak hangzik, ám a mi esetünkben, ahol az analízis *common garden* körülmények között felnőtt, második generációs egyedeken alapszik, a szisztematikus közvetlen környezeti hatások szerepe jelentéktelennek tekinthető. Ugyanakkor ismeretes, hogy a korai növekedés a legtöbb szövettípusban a sejtek számának, míg a későbbi szakaszban már inkább a méretének növekedéséből adódik (Atchley et al. 1984; Riska et al. 1984; Atchley & Zhu 1997), és így a későbbi szakaszban a random környezeti „zaj" szerepe igenis felerősödhet. A

vizsgálatunkban megfigyelt általános mintázat miszerint azonos genomrégiók több, a méretet és növekedést leíró változóval is kapcsolatot mutattak pleiotropikus hatásra vagy fizikailag közeli gének kapcsoltságára utalnak, bár logikus magyarázat az is, hogy több változónk valójában ugyanazt a tulajdonságot becsülte. Az eredményeink kiváló alapot nyújtanak későbbi, finomabb felbontású vizsgálatoknak, amelyek a releváns géneket azonosíthatják.

4.3.2.2.3 A viselkedés genetikai háttere

Noha a viselkedés hatása a rátermettségre nem kérdéses, a viselkedés genetikai háttere meglehetősen alulkutatott egyéb, főleg morfológiai, élettani vagy életmenet tulajdonságokhoz képest. Különböző viselkedési tulajdonságok gyakran örökölhetőek (van Oers et al. 2005), de a konkrét genetikai szabályozása ezeknek az extrém plaszticitást (West-Eberhard 2003) és tipikusan alacsony repetabilitást (Bell et al. 2009) mutató tulajdonságoknak ritkán feltárt természetes populációkban. Mivel a viselkedés változatosságának kialakításában az egyes gének szerepe általában alacsony (Boake et al. 2002; Mackay et al. 2009), a kandidáns gén megközelítés az időnkénti sikerek ellenére (pl. Fidler et al. 2007; Korsten et al. 2010) sem túl kecsegtető. Ezzel szemben az *a priori* várakozásokkal nem terhelt *QTL mapping* megközelítés nagyobb eséllyel képes lokalizálni a releváns géneket (Reif & Leish 2003; van Oers & Mueller 2010), újabban már természetes populációkban is (Wright et al. 2006a,b; Weber et al. 2013). A viselkedésért felelős gének gyakran a teljes genomban elszórva találhatóak (Henderson et al. 2004; Wright et al. 2006a,b), bár ismertek kivételek ahol komplex viselkedési mechanizmusokért egyetlen gén a felelős (Weber et al. 2013).

A testméret és növekedési ráta mellett egy másik általános tulajdonság ahol populációs szétválást találtunk a kilenctüskés pikónál a viselkedés volt (4.1.5; Herczeg et al. 2009b; Herczeg & Välimäki 2011): a kis tavi pikók aktívabbak, kockázatvállalóbbak és agresszívebbek voltak, mint a tengeri fajtársaik. A *QTL mapping* vizsgálatainkban szereplő F2 generációs izolált kis tó × tenger hibrid kilenctüskés pikóinknál az előbbiekben tárgyalt morfológiai és életmenet karaktereken kívül (4.3.2.2.1 és 4.3.2.2.2) mértünk még három viselkedési tulajdonságot is (Laine et al. 2014): táplálkozási aktivitást, kockázatvállalást táplálkozási kontextusban (innentől: kockázatvállalás) és kockázatvállalást explorációs kontextusban (innentől: exploráció). Ezeket a változókat analizáltuk külön-külön és egy főkomponens analízisből származó két főkomponenssel is. Az első főkomponens az egyedeket egy közös bátorság-tengelyen (*shyness-boldness continuum*) helyezte el, a második pedig elkülönítette a többi változótól független komponensét az explorációnak.

A két legerősebb jelet a viselkedési főkomponensek adták: a bátorság-főkomponens esetén a 3. kromoszómán található genomrégió a változatosság 7%-át magyarázta, az explorációs főkomponens esetén pedig 6%-ot magyarázott a 8. kromoszómán lévő genomrégió. A többi összefüggés gyengébb volt, az adott

tulajdonság 3-5%-át magyarázta. Az explorációs viselkedés az explorációs főkomponenssel azonos azonos genomikai régióval és a bátorság-főkomponenssel azonos kromoszómával, de valamennyire eltérő régióval is mutatott kapcsolatot. A táplálkozási aktivitás szintén az általános bátorság-főkomponenssel azonos kromoszómához, de valamennyire eltérő régióhoz kapcsolódott. A kockázatvállalás a 7. kromoszómával mutatott gyenge összefüggést. Az itt kiragadott kapcsolatokon kívül még több gyenge jelet is detektáltunk (részleteket lásd Laine et al. 2014 a Függelékben, 9). Az analízisünkben a statisztikai erő egy 10%-ot elérő hatás detektálására ≈ 1 volt, míg egy 2%-os hatás esetén is 0,4-es statisztikai erővel számolhattunk. Ezért ugyan elképzelhető, hogy néhány gyenge jelet nem mutattunk ki, de számottevő változatosságért felelős genomrégió szinte biztosan nem maradt detektálatlan. Összességében 4-20%-át magyarázták a feltárt genomrégiók a viselkedési változóknak. Ez nagyjából megfelel a várakozásoknak (Flint 2003; Flint & Munafo 2013) a rendkívül plasztikus (West-Eberhard 2003) és általában alacsony repetabilitást mutató (Bell et al. 2009) viselkedési tulajdonságoknál. A 3. kromoszóman az általános bátorság-főkomponenst befolyásoló genomrégió és a 8. kromoszóman azonosított, a többi viselkedéstől független explorációs komponenst befolyásoló genomrégió a legígéretesebb szakaszok a jövőbeli finom skálán végzett munkához, melynek eredményeképp az aktuálisan ható gének azonosíthatóak lesznek. A kandidáns viselkedési génekhez tervezett mikroszatellita markerek (Laine et al. 2012b) közül kettő (*Ppbig5*, *Ppbig8*) volt a hatással bíró genomrégiókon belül. Mindkét marker a 7. kromoszóman lévő, kockázatvállalással összefüggő genomrégióban helyezkedett el, és a Dopamin Receptor D1 (*Dopamine Receptor D1*, *Drd1*), illetve a Szerotonin Receptor 3B (*Serotonin Receptor 3b*, *Htr3b*) gének szerepét indukálta. A két gén szerepe ismert a viselkedés kialakításában (*Drd1*: agresszió kutyáknál, Våge et al. 2010; szaporodási viselkedés csirkénél, Xu et al. 2010; hiperaktivitás/autizmus embernél, Hettinger et al. 2008; *Htr3b*: antiszociális viselkedés embernél, Ducci et al. 2009) de a kilenctüskés pikók esetében kifejtett szerep tisztázása további vizsgálatokat igényel.

Érdekes, hogy amennyiben a jelen viselkedési és az előző fejezetben tárgyalt méretet/növekedést illető eredményeket (4.3.2.2.2; Laine et al. 2013) egyszerre vizsgáljuk, több átfedést is találunk a különböző tulajdonságokkal összefüggő genomrégiók között. Ez persze lehet a széles konfidencia intervallumokból eredő melléktermék, de elképzelhető pleiotrópikus hatás és fizikai kapcsoltság több gén között is. A *Pggm2* mikroszatellita markerhez közel eső genomrégió a 8. kromoszóman talán a legszembeötlőbb. Ez a régió befolyásolja az explorációt (exploráció: 4%; exploráció-főkomponens: 6%) és a méretet (testhossz 47 napos korban: 9%; növekedési konstans: 3%; növekedés a 2. stádiumban [2. mérés – 1. mérés]: 6%; növekedés a 3. stádiumban [3. mérés – 2. mérés]: 10%) is. A marker a *pituitary adenylate cyclase-activating polypeptide* (*ADCYAP1*/*ACAPRb*, magyar neve számomra ismeretlen) génhez volt tervezve. Ez a gén egyaránt szerepet kap a növekedésben (Lugo et al. 2008) és a posztraumatikus stresszben is (Ressler et al. 2011), tehát reális kandidáns egy viselkedés / növekedés pleiotrópikus hatást kiváltó

génre. A növekedést és viselkedést egyaránt befolyásoló genomrégiókra van példa más fajnál is (Schütz et al. 2004; Wirén et al. 2013). Az ultimális magyarázat a közös hatással bíró gén evolúciós szerepére aránylag egyszerű: a nagy testméret és gyors növekedés egyaránt magas energiaigényű és ezért magasabb viselkedési aktivitáshoz kötött. További átfedéseket lásd: Laine et al. (2014) a Függelékben (9).

4.3.3 Összegzés

Dolgozatom harmadik, utolsó fejezetében az első fejezetben feltárt főbb habitat-függő populációs szétválást mutató tulajdonságok genetikai hátterét vizsgáltuk több megközelítésben. A populációs hibridek fenotípusos analízise bizonyította a testméret, ivarérés ideje és a táplálkozási aktivitás populációs különbségei mögötti genetikai mechanizmusok meglétét. Additív genetikai (testméret) és domináns genetikai (ivarérés ideje, táplálkozási aktivitás) mechanizmusokra is találtunk példát. A speciális izolált kis tavi környezet támasztotta szelekciós erők erősségét jelzi, hogy recesszív tulajdonságok is uralkodóvá válhattak ebben a környezetben. Többször figyeltünk meg aszimmetrikus anyai hatást: a kis tavi nőstények valami módon befolyásolták az utódaik fenotípusát, a genetikai szétválást tovább erősítve.

Amíg a *genome scan* módszerrel nem sikerült a kis tavi adaptációban fontos genomrégiókat azonosítani a kis tavi populációk extrém alacsony genetikai diverzitása miatt, a *QTL mapping* megközelítés sikeres volt. A hasi tüske és a mell-öv redukciójában meghatározó genomszakaszként azonosítottuk a *Pitx1* gént. Ez megegyezett a háromtüskés pikónál talált eredményekkel, de eltért az észak-amerikai kilenctüskés pikó kládban tapasztaltaktól, bizonyítván, hogy a taxonómiai távolság nem feltétlenül prediktálja a genetikai mechanizmusok hasonlóságát. A testméret/növekedés és a viselkedési változók tekintetében is a „sok gén kis hatással” mintázat nyert bizonyítást. Az eredmények alapján több kandidáns genomikai régiót lehet majd finomabb skálán vizsgálni és a releváns géneket azonosítani. Elképzelhető olyan gén is mely pleiotrópikus hatást fejt ki a méretre és viselkedésre.

5. Összefoglalás és kitekintés

A dolgozatomban összefoglalt vizsgálatok eredményeképpen a kilenctüskés pikót mára, mint ígéretes evolúcióbiológiai és genetikai modellfajt tarthatjuk számon (Merilä 2013). Több, geográfiailag és genetikailag is izolált kis tóban (Svédországban, Finnországban és Oroszországban vannak ilyen ismert populációk) egymástól függetlenül egy hasonló fenotípus evolválódott: az ezeken az élőhelyeken élő pikókat óriás testméret (akár kétszeres hossz, hatszoros tömeg), hosszú növekedési időszak, késleltetett ivarérés, megnövekedett fekunditás, redukálódott morfológiai védelmi struktúrák, aktív/bátor viselkedési típus és a csoportos élet magas költségei jellemzik, valamint neurobiológiai és érzékszervi tulajdonságokban is szisztematikusan eltérnek a „közönséges” tengeri, nagy tavi vagy folyóvízi

fajtársaiktól. Ezek a többszörös független evolúciós események már magukban is, illetve az evolúciós szupermodellnek tekintett közeli rokon háromtüskés pikóval (Gibson 2005; Heins 2012) való összevetésben is kiváló rendszert nyújtanak a hasonló környezetekhez való ismételt alkalmazkodás evolúciós hátterének a tanulmányozásához különböző evolúciós időskálákon. Ilyen szituációkban legalább három mechanizmus képzelhető el: paralel evolúció (azonos fenotípusos válasz azonos genetikai háttérrel), konvergens evolúció (azonos fenotípusos válasz eltérő genetikai háttérrel) és a funkcionális ekvivalencia elvén működő evolúció (alternatív fenotípusos válasz értelemszerűen eltérő genetikai háttérrel). Ezek relatív fontossága, az előfordulásukat valószínűsítő tényezők, és az aktuális mechanizmusok pontos feltérképezése a kurrens evolúcióbiológia aktuális kérdései. A funkcionális genomika jelenleg zajló módszertani „robbanása" és az ebből következően várható szemléletbeli paradigmaváltás (az evolúciót nem csak közvetetten a fenotípus szintjén, hanem közvetlen módon a genomban is rutinszerűen lehet majd vizsgálni) idejében különösen fontos az ilyen és ehhez hasonló modellrendszerek feltárása, hiszen a módszertan magában még nem garantál tudományos áttörést. Szükség van tehát megfelelően leírt evolúciós modellrendszerekre ahol a természetes mintázatok példaértékűek és ugyanakkor a genomikai vizsgálatok feltételei is adottak. A dolgozatomban ismertetett rendszer genomikai feldolgozása megkezdődött (ennek kezdeti eredményeit mutattam be a 4.3.2 fejezetben). A következő években a területet meghatározó eredmények várhatóak a lokális adaptációk genetikai mechanizmusait illetően.

A vizsgálataim alanyától és a konkrét eredményektől függetlenül fontosnak tartom a dolgozatban alkalmazott megközelítés szerepének kiemelését is. Sok ökológiailag releváns, a rátermettséget befolyásoló kvantitatív tulajdonság esetében is két fő pilléren nyugszik a tudásunk, egyrészt a fajok vagy magasabb taxonómiai szintek összehasonlításán, másrészt pedig a populációkon belüli egyedi szintű vizsgálatokon. E két megközelítés között méltatlanul háttérbe szorulnak a fajon belüli populációs összehasonlítások. Ez nem szerencsés, hiszen a faj feletti szinten végzett vizsgálatok természetükből adódóan korrelatívak, és így bizonyító erővel nem rendelkeznek, a populáción belüli mintázatokból pedig csak extrapolálni tudjuk (leszámítva a sokgenerációs hosszútávú vizsgálatokat) az evolúciós eseményeket. Ezzel szemben a populációs összehasonlításoknál jó eséllyel azonosítani tudjuk a lokális adaptációkért felelős környezeti tényezőket és a populációk evolúciós története is rekonstruálható. Kvantitatív genetikai kísérletekben pedig a genetikailag meghatározott mintázatok elválaszthatók a környezet közvetlen hatásaitól, illetve a genetikai komponens esetén tesztelni tudjuk a természetes szelekció szerepét a random genetikai sodródáshoz hasonlítva. A dolgozatomban ismertetett vizsgálatok között több is úttörőnek tekinthető a populációs összehasonlítások alkalmazásának tekintetében, pl. agy struktúra, érzékszervek, és a fenotipikus plaszticitás populációs összehasonlítása is egy gyümölcsöző megközelítés, ami több figyelmet érdemelne. Reményeim szerint az eredményeink más kutatókat is ösztönöznek majd a

populációs összehasonlításokban rejlő lehetőségek kihasználására a klasszikus evolúciós megközelítéssel még nem vizsgált tulajdonságok esetében is.

Végezetül meg kell említenem, hogy a Fennoskandináv kilenctüskés pikó rendszer ideális lenne az ökológiai fajképződés számos kérdésének vizsgálatához. Bár ezirányú vizsgálatokat célzottan nem végeztem, a megfigyeléseim alapján mind pre-, mind posztzigotikus izoláció is jó eséllyel jelen van a kis tavi és a többi (tengeri, nagy tavi, folyóvízi, stb.) populációk között. A kis tavi fenotípus ismételt evolúciója egy érdekes szituációt teremtett: elképzelhető, hogy a teljesen függetlenül kialakult kis tavi populációk egymástól nem, de a többi populációtól szexuálisan izoláltak. Reményeim szerint ilyen irányú vizsgálatok a fentebb tárgyalt evolúciós genomikai vizsgálatokkal párhuzamosan fognak zajlani, a két összefüggő kérdéskör leghatékonyabb megközelítése érdekében.

6. Köszönetnyilvánítás

A dolgozatomban bemutatott kutatásokban két ember segítsége volt nélkülözhetetlen. Juha Merilä Professzor Úrnak hálával tartozom, amiért meghívott a kutatócsoportjába (Ecological Genetics Research Unit (EGRU], University of Helsinki) ahol hat évet töltöttem, és akivel a mai napig együttműködünk. A kiindulópontunkhoz képest, miszerint „Gábor, Kuusamo környékén állítólag furán néznek ki a pikók, nincs kedved fogni párat?”, elég messzire jutottunk, és ebben fontos szerepe volt Juha Merilä nem szűnő lelkesedésének, nyitottságának és teljes körű támogatásának. Dr. Gonda Abigél segítsége nélkül szintén nem jutottam volna idáig, nélkülözhetetlen munkatárs volt a legtöbb vizsgálatban, a halak gyűjtésétől kezdve a publikációk megírásáig. Segítségét és társaságát nem lehet eléggé megköszönni.

Gonda Abigélén kívül még három volt PhD diák munkája volt jelentős, köszönöm Dr. Kaisa Välimäki, Dr. Veronica Laine és Dr. Nurul Izza ab Ghani elhivatottságát, amivel részt vettek a kutatásaimban. Dr. Nina Trokovic, Dr. Wolfgang Waser, Dr. Pia Saarinen, Dr. Takahito Shikano, Prof. Kristian Donner és Prof. Craig Primmer együttműködése is sokat segített egyes vizsgálatok kivitelezésében. Az EGRU több tagjának is hálával tartozom, amiért mindig készen álltak szakmai megbeszélésekre és a gyakorlati problémák megoldására az akvatechnikától a statisztikáig, köszönöm Dr. Tuomas Leinonen, Dr. Jose Manuel Cano Arias, Dr. Chikako Matsuba, és Dr. Scott McCairns segítségét. Johanna Paananen, Simo Rintakosi és Mirva Turtiainen a kísérletekben, Marika Karjalainen pedig a genetikai vizsgálatokban nyújtott segítséget. A terepi munka nem valósulhatott volna meg Prof. Pirkko Siikämäki (Oulanka Resarch Station, Finnország) és Prof. Victor Berger (White Sea Biological Station, Oroszország) együttműködése nélkül. Vizsgálataimat anyagilag a Finn Akadémia (*Academy of Finland*), a dolgozat megírását pedig a Magyar Tudományos Akadémia Bólyai János Tudományos Ösztöndíja támogatta.

Tágabb értelemben hálával tartozom MSc és PhD témavezetőimnek, Dr. Korsós Zoltánnak és Prof. Török Jánosnak amiért tanácsaikkal és példájukkal segítették a kutatóvá válásomat és amiért a mai napig szívesen vitatnak meg velem szakmai (és egyéb) problémákat. Sokat köszönhetek még az Eötvös Loránd Tudományegyetem Állatrendszertani és Ökológiai Tanszékén működő Viselkedésökológiai Csoportnak, ami egy optimális kutatói környezetet biztosított PhD diák koromtól egészen mostanáig. Dr. Hettyey Attila, Dr. Hegyi Gergely, Dr. Rosivall Balázs és Dr. Garamszegi László Zsolt azok a kollégák, akikkel sok időt töltöttem különböző problémák és eredmények megvitatásával, és akik mindig pozitívan hatottak rám.

Végezetül szeretném megköszönni feleségemnek, Abigélnek és a fiaimnak, Gábornak és Áronnak, amiért a legelvakultabb kutatói pillanatokban is képesek emlékeztetni arra, hogy mi is az igazán fontos az életben.

dc_892_14

7. Az értekezés alapjául szolgáló saját közlemények listája

Folyóiratcikkek

AB GHANI NI, HERCZEG G, MERILÄ J (2012) Body size divergence in nine-spined sticklebacks: disentangling additive genetic and maternal effects. **Biological Journal of the Linnean Society** 107:521-528

AB GHANI NI, HERCZEG G, MERILÄ J (2013) Evidence for genetic differentiation in timing of maturation among nine-spined stickleback populations. **Journal of Evolutionary Biology** 26:775-782

AIKIO S, HERCZEG G, KUPARINEN A, MERILÄ J (2013) Optimal growth strategies under divergent predation pressure. **Journal of Fish Biology** 82:318-331

BRUNEAUX M, JOHNSTON SE, HERCZEG G, MERILÄ J, PRIMMER CR, VASEMÄGI A (2013) Molecular evolutionary and population genomic analysis of the nine-spined stickleback using a modified RAD tag approach. **Molecular Ecology** 22:565-582

GONDA A, HERCZEG G, MERILÄ J (2009) Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? **Journal of Evolutionary Biology** 22:1721-1726

GONDA A, HERCZEG G, MERILÄ J (2009) Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain. **Proceedings of the Royal Society B** 276:2085-2092

GONDA A, HERCZEG G, MERILÄ J (2011) Population variation in brain size of nine-spined sticklebacks (*Pungitius pungitius*) – local adaptation or environmentally induced variation? **BMC Evolutionary Biology** 11:75

GONDA A, VÄLIMÄKI K, HERCZEG G, MERILÄ J (2012) Brain development and predation: plastic responses depend on evolutionary history. **Biology Letters** 8:249-252

HERCZEG G, VÄLIMÄKI K (2011) Intraspecific variation in behaviour: effects of evolutionary history, ontogenetic experience and sex. **Journal of Evolutionary Biology** 24:2434-2444

HERCZEG G, GONDA A, MERILÄ J (2009) Evolution of gigantism in nine-spined stickleback. **Evolution** 63:3190-3200

HERCZEG G, GONDA A, MERILÄ J (2009) Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). **Journal of Evolutionary Biology** 22:544-552

HERCZEG G, GONDA A, MERILÄ J (2009) The social cost of shoaling covaries with predation risk in nine-spined stickleback (*Pungitius pungitius*) populations. **Animal Behaviour** 77:575-580

HERCZEG G, GONDA A, MERILÄ J (2010) Rensch's rule inverted – female-driven gigantism in nine-spined stickleback (*Pungitius pungitius*). **Journal of Animal Ecology** 79:581-588.

HERCZEG G, TURTIAINEN M, MERILÄ J (2010) Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. **Biological Journal of the Linnean Society** 101:403-416

HERCZEG G, AB GHANI NI, MERILÄ J (2013) Evolution of stickleback feeding behaviour: genetics of population divergence at different ontogenetic stages. **Journal of Evolutionary Biology** 26:955-962

HERCZEG G, GONDA A, KUPARINEN A, MERILÄ J (2012) Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? **Evolutionary Ecology** 26:109-122

HERCZEG G, VÄLIMÄKI K, GONDA A, MERILÄ J (2014) Evidence for sex-specific selection in brain: a case study of the nine-spined stickleback. **Journal of Evolutionary Biology** 27:1604-1612

KARHUNEN M, OVASKAINEN O, HERCZEG G, MERILÄ J (2014) Bringing habitat information into statistical tests of local adaptation in quantitative traits: a case study in nine-spined sticklebacks. **Evolution** 68:559-568

LAINE V, PRIMMER C, HERCZEG G, MERILÄ J, SHIKANO T (2012) Isolation and characterization of 13 new nine-spined stickleback, *Pungitius pungitius*, microsatellites located nearby candidate genes for behavioural variation. **Annales Zoologici Fennici** 49:123-128

LAINE VN, SHIKANO T, HERCZEG G, VILKKI J, MERILÄ J (2013) Quantitative trait loci for growth and body size in the nine-spine stickleback *Pungitus pungitius* L. **Molecular Ecology** 22: 5861-5876

LAINE VN, HERCZEG G, SHIKANO T, VILKKI J, MERILÄ J (2014) QTL analysis of behavior in nine-spined sticklebacks (*Pungitius pungitius*). **Behavior Genetics** 44:77-88

SAARINEN P, PAHLBERG J, HERCZEG G, VILJANEN M, KARJALAINEN M, MERILÄ J, DONNER K (2012) Spectral tuning by selective chromophore uptake in rods and cones of eight populations of nine-spined stickleback (*Pungitius pungitius*). **Journal of Experimental Biology** 215:2760-2773

SHIKANO T, SHIMADA Y, HERCZEG G, MERILÄ J (2010) History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. **Molecular Ecology** 19:1147-1161

SHIKANO T, LAINE VN, HERCZEG G, VILKKI J, MERILÄ J (2013) Genetic architecture of parallel pelvic reduction in ninespine sticklebacks. **Genes, Genomes, Genetics** 3:1833-1842

TROKOVIC N, HERCZEG G, MCCAIRNS RJS, AB GHANI NI, MERILÄ J (2011) Intraspecific divergence in the lateral line system in the nine-spined stickleback (*Pungitius pungitius*). **Journal of Evolutionary Biology** 24:1546-1558

TROKOVIC N, HERCZEG G, AB GHANI NI, SHIKANO T, MERILÄ J (2012) High levels of fluctuating asymmetry in isolated stickleback populations. **BMC Evolutionary Biology** 12:115

VÄLIMÄKI K, HERCZEG G (2012) Ontogenetic and evolutionary effects of predation and competition on nine-spined stickleback *(Pungitius pungitius)* body size. **Journal of Animal Ecology** 81:859-867

VÄLIMÄKI K, HERCZEG G, MERILÄ J (2012) Morphological antipredator defences in the nine-spined stickleback: constitutive, induced or both? **Biological Journal of the Linnean Society** 107:854-866

Publikálatlan kéziratok

TURTIAINEN M, HERCZEG G, MERILÄ J: Sexual dimorphism in nine-spined stickleback (*Pungitius pungitius*) body shape and armour. **Kézirat**

VÄLIMÄKI K, HERCZEG G, MERILÄ J: The effects of food supply and perceived predation risk on the energy reserves of nine-spined sticklebacks from ecologically divergent populations. **Kézirat**

VÄLIMÄKI K, HERCZEG G, TROKOVIC N, MERILÄ J: Local adaptation and phenotypic plasticity in the lateral line organs – an experiment. **Kézirat**

dc_892_14

8. Irodalomjegyzék

Ab Ghani NI, Herczeg G, Merilä J (2012) Body size divergence in nine-spined sticklebacks: disentangling additive genetic and maternal effects. Biol J Linn Soc 107:521–528.

Ab Ghani NI, Herczeg G, Leinonen T, Merilä J (2013) Evidence for genetic differentiation in timing of maturation among nine-spined stickleback populations. J Evol Biol 26:775-782.

Abrams PA, Rowe L (1996) The effects of predation on the age and size of maturity of prey. Evolution 50:1052–1061.

Adar E, Lotem A, Barnea A (2008) The effect of social environment on singing behavior in the zebra finch (*Taeniopygia guttata*) and its implication for neuronal recruitment. Behav Brain Res 187:178–184.

Ådjers K, Appelberg M, Eschbaum R, Lappalainen A, Minde A, Repecka R, Thoresson G (2006) Trends in coastal fish stocks of the Baltic Sea. Boreal Environment Research 11:13–25.

Aiello LC, Wheeler P (1995) The expensive tissue hypothesis – The brain and digestive system in human and primate evolution. Curr Anthrop 36:199-221.

Aikio S, Herczeg G, Kuparinen A, Merilä J (2013) Optimal growth strategies under divergent predation pressure. J Fish Biol 82:318-331.

Airey DC, DeVoogd TJ (2000) Greater song complexity is associated with augmented song system anatomy in zebra finches. Neuroethol 11:2339-2344.

Aldenhoven JT, Miller MA, Corneli PS, Shapiro MD (2010) Phylogeography of ninespine sticklebacks (*Pungitius pungitius*) in North America: glacial refugia and the origins of adaptive traits. Mol Ecol 19:4061–4076.

Alho JS, Herczeg G, Söderman F, Laurila A, Jönsson KI, Merilä J (2010) Increasing melanism along a latitudinal gradient in a widespread amphibian: local adaptation, ontogenic or environmental plasticity? BMC Evol Biol 10:317.

Ali M, Wootton RJ (1999) Effect of variable food levels on reproductive performance of breeding female three-spined sticklebacks. J Fish Biol 55:1040–1053.

Allan MF, Eisen EJ, Pomp D (2005) Genomic mapping of direct and correlated responses to long-term selection for rapid growth rate in mice. Genetics 170:1863–1877.

Allen JRM, Wootton RJ (1982) Effect of food on the growth of carcase, liver and ovary in female *Gasterosteus aculeatus* L. J Fish Biol 21:537–547.

Alroy J (1998) Cope's rule and the dynamics of body mass evolution in North American fossil mammals. Science 280:731-734.

Andersson M (1994) Sexual Selection. Princeton University Press. Princeton, New Jersey

Andersson L, Georges M (2004) Domestic-animal genomics: deciphering the genetics of complex traits. Nat Rev Genet 5:202–212.

Arendt JD, Reznick DN (2005) Evolution of juvenile growth rates in female guppies (*Poecilia reticulata*): predator regime or resource level? Proc R Soc B 272:333–337.

Atchley WR (1984) Ontogeny, timing of development, and genetic variance-covariance structure. Am Nat 123:519–540.

Atchley WR, Zhu J (1997) Developmental quantitative genetics, conditional epigenetic variability and growth in mice. Genetics 147:765–776.

Atchley WR, Riska B, Kohn LAP, Plummer AA, Rutledge JJ (1984) A quantitative genetic analysis of brain and body size associations, their origin and ontogeny: data from mice. Evolution 38:1165–1179.

Auld JR, Agrawal AA, Relyea RA (2010) Re-evaluating the costs and limits of adaptive phenotypic plasticity. Proc R Soc B 277:503–511.

Aviles JM, Garamszegi LZ (2007). Egg rejection and brain size among potential hosts of the common cuckoo. Ethology 113:562–572.

Baker JA, Wund MA, Chock RY, Ackein L, Elsemore R, Foster SA (2010) Predation history and vulnerability: Conservation of the stickleback adaptive radiation. Biological Conservation 143:1184–1192.

Bănărescu PM, Paepke H-J (2001) The Freshwater Fishes of Europe. Vol. 5/III. AULA-Verlag, Wiebelsheim

Barton RA, Harvey P H (2000) Mosaic evolution of brain structure in mammals. Nature 405:1055–1058.

Barton RA, Capellini I (2011) Maternal investment, life history, and the costs of brain growth in mammals. Proc Natl Acad Sci USA 108:6169-6174.

Barton RA, Purvis A, Harvey PH (1995) Evolutionary radiation of visual and olfactory brain systems in primates, bats and insectivores. Phil Trans R Soc Lond B 348: 381–392.

Beldade P, Mateus ARA, Keller RA (2011) Evolution and molecular mechanisms of adaptive developmental plasticity. Mol Ecol 20:1347–1363.

Bell G (1980). The costs of reproduction and their consequences. Am Nat 116:45–76.

Bell AM (2005) Behavioural differences between individuals and two populations of stickleback (*Gasterosteus aculeatus*). J Evol Biol 18:464–473.

Bell AM (2007) Future directions in behavioural syndrome research. Proc R Soc B 274:755–761.

Bell AM, Hankison SJ, Laskowski KL (2009) The repeatability of behaviour: a meta-analysis. Anim Behav 77:771–783

Bell MA, Foster SA (1994) The evolutionary biology of the three-spine stickleback. Oxford University Press, New York

Bell MA, Steward JD, Park P (2009) The world's oldest fossil threespine stickleback fish. Copeia 2009:256–265.

Bell MA, Ortí G, Walker JA, Koenings JP (1993) Evolution of pelvic reduction in threespine stickleback fish—a test of competing hypotheses. Evolution 47:906–914.

Bell AM, Dingemanse NJ, Hankison SJ, Langenhof MBW, Rollins K. (2011) Early exposure to nonlethal predation risk by size-selective predators increases somatic growth and decreases size at adulthood in threespined sticklebacks. J Evol Biol 24:943–953.

Bergstrom CA (2002) Fast-start swimming performance and reduction in lateral plate number in threespine stickleback. Can J Zool 80:207–213.

Bergstrom CA, Reimchen TE (2003) Asymmetry in structural defenses: insights into selective predation in the wild. Evolution 57:2128–2138.

Berrigan D, Charnov EL (1994) Reaction norms for age and size at maturity in response to temperature: a puzzle for life historians. Oikos 70:474–478.

Berry JF, Shine R (1980) Sexual dimorphismand sexual selection in turtles (order Testudines). Oecologia 44:185–191.

von Bertalanffy L (1938) A quantitative theory of organic growth (inquiries on growth laws II). Human Biol 10:181–213.

Biro PA, Post JR, Abrahams MV (2005) Ontogeny of energy allocation reveals selective pressure promoting risk-taking behaviour in young fish cohorts. Proc Roy Soc B 272:1443–1448.

Biro PA, Abrahams MV, Post JR, Parkinson EA (2004) Predators select against high growth rates and risktaking behaviour in domestic trout populations. Proc R Soc Lond B 271:2233–2237.

Biro PA, Abrahams MV, Post JR, Parkinson EA (2006) Behavioural trade-offs between growth and mortality explain evolution of submaximal growth rates. J Anim Ecol 75:1165–1171.

Birse SC, Leonard RB, Coggeshall RE (1980) Neuronal increase in various areas of the nervous system of the guppy, Lebistes. J Comp Neurol 194:291-301.

Blanckenhorn WU (2000) The evolution of body size: what keeps organisms small? Q Rev Biol 75:385–407.

Blanckenhorn WU (2005) Behavioural causes and consequences of sexual size dimorphism. Ethology 111:977–1016.

Blanckenhorn WU, Demont M (2004) Bergmann and converse Bergmann latitudinal clines in arthropods: two ends of a continuum? Integr Comp Biol 44:413–424.

Blaxter JHS, Fuiman LA (1990) The role of the sensory systems of herring larvae in evading predatory fishes. J Mar Biol 70: 413–427.

Blouw DM, Boyd GJ (1992) Inheritance of reduction, loss, and asymmetry of the pelvis in *Pungitius pungitius* (ninespine stickleback). Heredity 68:33-42.

Blumstein DT (2006) The multi-predator hypothesis and the evolutionary persistence of antipredator behavior. Ethology 112: 209–217.

Blumstein DT, Daniel JC (2003) Foraging behaviour of three Tasmanian macropodid marsupials in response to present and historical predation threat. Ecography 26:585–594.

Boake CRB, Arnold SJ, Breden F, Meffert LM, Ritchie MG, Taylor BJ, Wolf JB, Moore AJ (2002) Genetic tools for studying adaptation and the evolution of behavior. Am Nat 160:S143–S159.

Bolnick DI, Preisser EL (2005) Resource competition modifies the strength of trait-mediated predatorprey interactions: a meta-analysis. Ecology 86:2771–2779.

Bolnick DI, Lau OL (2008) Predictable patterns of distruptive selection in stickleback in postglacial lakes. Evolution 172:1–11.

Bookstein FL (1991) Morphometric Tools for Landmark Data: Geometry and Biology. Cambridge University Press, Cambridge.

Botham MS, Krause J (2005) Shoals receive more attacks from the wolf-fish (*Hoplias malabaricus* Bloch, 1794). Ethology 111:881–890.

Boughman J W (2007) Speciation in sticklebacks. in: Östlund-Nilsson S, Mayer I, Huntingford FA (eds.) Biology of the three-spined stickleback. CRC Press, Boca Raton, FL. pp 83–126

Breden F, Scott MA, Michel E (1987) Genetic differentiation for anti-predator behaviour in the Trinidad guppy, *Poecilia reticulata*. Anim Behav 35:618–620.

Bridges CDB (1972) The rhodopsin-porphyropsin visual system. In: Dartnall HJA (ed) Handbook of Sensory Physiology VII/1: Photochemistry of Vision. Springer-Verlag, Berlin, pp 417- 480

Broglio C, Rodriguez F, Salas C (2003) Spatial cognition and its neural basis in teleost fish. Fish Fish 4: 247–255.

Brönmark C, Miner JG (1992) Predator-induced phenotypical change in body morphology in crucian carp. Science 258:1348–1350.

Brönmark C, Pettersson LB (1994) Chemical cues from piscivores induce a change in morphology in crucian carps. Oikos 70:396–402.

Brown C, Warburton K (1997) Predation recognition and anti-predator responses in the rainbowfish Melanotaenia eachamensis. Behav Ecol Sociobiol 41:61–68.

Brown C, Jones F, Braithwaite VA (2005) In situ examination of boldness-shyness traits in the tropical poeciliid, *Brachyraphis episcope*. Anim Behav 70:1003–1009.

Brown C, Burgess F, Braithwaite VA (2007) Heritable and experiential effects on boldness in a tropical poeciliid. Behav Ecol Sociobiol 62:237–243.

Brydges NM, Colegrave N, Heathcote RJP, Braithwaite VA (2008) Habitat stability and predation pressure affect temperament behaviours in populations of three-spined sticklebacks. J Anim Ecol 77:229–235.

Bruneaux M, Johnston SE, Herczeg G, Merilä J, Primmer CR, Vasemägi A(2013) Molecular evolutionary and population genomic analysis of the nine-spined stickleback using a modified

restriction site-associated DNA tag approach. Mol Ecol 22:565–582.

Bulmer M (1994) Theoretical Evolutionary Ecology. Sinauer Associates, Sunderland, MA

Burns JG, Saravanan A, Rodd FH (2009) Rearing environment affects the brain size of guppies: lab-reared guppies have smaller brains than wild-caught guppes. Ethology 115:122-133.

Van Buskirk J, Schmidt BR (2000) Predator-induced phenotypic plasticity in larval newts: tradeoffs, selection, and variation in nature. Ecology 81:3009–3028.

Van Buskirk J, Arioli M (2005) Habitat specialization and adaptive phenotypic divergence of anuran populations. J Evol Biol 18:596–608.

Cahn PH, Shaw E, Atz EH (1968) Lateral line histology as related to the development of schooling in the atherinid fish. Menidia Bull Mar Sci 18:660–670.

Calow P (1982) Homeostasis and fitness. Am Nat 120:416–419.

Carlborg O, Kerje S, Sch€utz K, Jacobsson L, Jensen P, Andersson L (2003) A global search reveals epistatic interaction between QTL for early growth in the chicken. Genome Research. 13:413–421.

Carton AG, Montgomery JC (2004) A comparison of lateral line morphology of blue cod and torrentfish: two sandperches of the family Pinguipedidae. Environ Biol Fishes 70:123–131.

Casselman SJ, Schulte-Hostedde AI (2004) Reproductive roles predict sexual dimorphism in internal and external morphology of lake whitefish, *Coregonus clupeaformis*. Ecol Freshw Fish 13:217–222.

Chan YF, Marks ME, Jones FC, Villarreal G Jr, Shapiro MD, Brady SD, Southwick AM, Absher DM, Grimwood J, Schmutz J, Myers RM, Petrov D, Jónson B, Schluter D, Bell MA, Kingsley DM (2010) Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a *Pitx1* enhancer. Science 15:302–305.

Chapman BB, Morrell LJ, Tosh C R, Krause J (2010) Behavioural consequences of sensory plasticity in guppies. Proc Roy Soc B 277:1395–1401.

Chapuis E, Martin G, Goudet J (2008) Effects of selection and drift on G matrix evolution in a heterogeneous environment: a multivariate Q(st)-F(st) test with the freshwater snail *Galba truncatula*. Genetics 180:2151-2161.

Charnov EL (1993) Life-History Invariants. Oxford University Press, Oxford

Charnov EL, Schaffer WM (1973) Life-history consequences of natural selection: Cole's results revisited. Am Nat 107:791–792.

Chellappa S, Huntingford FA, Strang RHC, Thomson RY (1989) Annual variation in energy reserves in male three-spined stickleback (*Gasterosteus aculeatus*) L. (Pisces, Gasterosteidae). J Fish Biol 35:275–286.

Chellappa S, Huntingford FA, Strang RHC, Thomson RY (1995) Condition factor and hepatosomatic index as estimates of energy status in male three-spined stickleback. J Fish Biol 47:775–787.

Cheverud JM, Routman EJ, Duarte FAM, van Swinderen B, Cothran K, Perel C (1996) Quantitative trait loci for murine growth. Genetics 142:1305–1319.

Chivers DP, Zhao X, Brown GE, Marchant TA, Ferrari MCO (2007) Predator-induced changes in morphology of a prey fish: the effects of food level and temporal frequency of predation risk. Evol Ecol 22:561–574.

Clarke BC (1975) The contribution of ecological genetics to evolutionary theory: detecting the direct effects of natural selection on particularly polymorphic loci. Genetics 79(Suppl.):101–113.

Clarke GM (1995) Relationships between developmental stability and fitness: application for conservation biology. Cons Biol 9:18–24.

Cole LC (1954) The population consequences of life history phenomena. Q Rev Biol 29:103–137.

Cole NJ, Tanaka M, Prescott A, Tickle CA (2003) Expression of limb initiation genes and

clues to the basis of morphological diversification in threespine sticklebacks. Curr Biol R951-R952

Colosimo PF, Peichel CL, Nereng K, Blackman BK, Shapiro MD, Schluter D, Kingsley DM (2004) The genetic architecture of parallel armor plate reduction in threespine sticklebacks. PLoS Biol 2:635–641.

Colosimo PF, Hosemann KE, Balabhadra S, Villarreal G, Dickson M, Grimwood J, Schmutz J, Myers RM, Schluter D, Kingsley DM (2005)Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. Science 307:1928–1933.

Conover DO, Schultz ET (1995) Phenotypic similarity and the evolutionary significance of countergradient variation. Trends Ecol Evol 10:248–252.

Coombs S, Janssen J, Webb JF (1988) Diversity of lateral line systems, evolutionary and functional considerations. In: Atema J, Fay RR, Popper AN, Tavolga WN (eds.) Sensory Biology of Aquatic Animals. Springer-Verlag, Heidelberg. pp 553–593

Coombs S, Finneran J, Conley RA (2000) Hydrodynamic imaging by the lateral line system of the Lake Michigan mottled sculpin. Phil Trans R Soc Lond 355:1111–1114.

Cope ED (1887) The Origin of the Fittest. Appleton, New York

Coss RG (1999) Effects of relaxed natural selection on the evolution of behavior. In: Foster SA, Endler JA (eds.) Geographic Variation in Behavior: Perspectives on Evolutionary Mechanisms. Oxford University Press, Oxford. pp 180–208

Côté IM, Poulin R (1995) Parasitism and group size in social animals: a metaanalysis. Behav Ecol 6:159–165.

Coyle SM, Huntingford FA, Peichel CL (2007) Parallel evolution of Pitx1 underlies pelvic reduction in Scottish threespine tickleback (*Gasterosteus aculeatus*). J Hered 98:581–586.

Cresko WA, Amores A, Wilson C, Murphy J, Currey M, Phillips P, Bell MA, Kimmel CB, Postlethwait JH (2004) Parallel genetic basis for repeated evolution of armor loss in Alaskan threespine stickleback populations. Proc Natl Acad Sci USA 101:6050–6055.

Crispo E (2007) The Baldwin effect and genetic assimilation: revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. Evolution 61:2469–2479

Crispo E (2008) Modifying effects of phenotypic plasticity on interactions among natural selection, adaptation and gene flow. J Evol Biol 21:1460–1469.

Crispo E, Chapman LJ (2010a) Geographic variation in phenotypic plasticity in response to dissolved oxygen in an African cichlid fish. J Evol Biol 23:2091-2103.

Crispo E, Chapman LJ (2010b) Hypoxia drives plastic divergence in cichlid body shape. Evol Ecol 25:949–964.

Cronin TW, Caldwell RL, Marshall J (2010) Tunable colour vision in a mantis shrimp. Nature 411:2000–2001.

Dangles O, Irschick D, Chittka L, Casas J (2009) Variability in sensory ecology: expanding the bridge between physiology and evolutionary biology. Q Rev Biol 84: 51–74.

Day T, Rowe L (2002) Developmental thresholds and the evolution of reaction norms for age and size at maturity. Am Nat 159:338–350.

DeFaveri J, Shikano T, Shimada Y, Goto A, Merilä J (2011) Global analysis of genes involved in freshwater adaptation in threespine sticklebacks (*Gasterosteus aculeatus*). Evolution 65:1800–1807.

DeWitt T, Sih A, Wilson DS (1998) Costs and limits of phenotypic plasticity. Trends Ecol Evol 13:77–81.

DeWoody JA, Avise JC (2000) Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. J Fish Biol 56:461–473.

Diamond MC, Law F, Rhodes H, Lindner B, Rosenzweig MR, Krech D, Bennett EL (1966)

Increases in cortical depth and glia numbers in rats subjected to enriched environment. J Comp Neurol 128:117–126.

Dijkgraaf S (1963) The functioning and significance of the lateral line organs. Biol Revs 38:51–105.

Dingemanse NJ, Kazem AJN, Réale D, Wright J (2010) Behavioural reaction norms: animal personality meets individual plasticity. Trends Ecol Evol 25:81-89.

Dmitriew CM (2011) The evolution of growth trajectories: what limits growth rate? Biol Rev 86:97–116.

Domenici P, Turesson H, Brodersen J, Brönmark C (2008) Predator-induced morphology enhances escape locomotion in crucian carp. Proc Roy Soc B 275:195–201.

Van Dongen S (2006) Fluctuating asymmetry and developmental instability in evolutionary biology: past, present and future. J Evol Biol 19:1727–1743.

Donner J (1995) The Quaternary History of Scandinavia. Cambridge University Press, Cambridge

Draganski B, May B (2008) Training-induced structural changes in the adult human brain. Behav Brain Res 192:137-142.

Ducci F, Enoch M-A, Yuan Q, Shen PH, White KV, Hodgkinson C, Albaugh B, Virkkunen M, Goldman D (2009) HTR3B is associated with alcoholism with antisocial behavior and alpha EEG power—an intermediate phenotype for alcoholism and comorbid behaviors. Alcohol 43:73–84.

Dunbar RIM, Shultz S (2007) Evolution in the social brain. Science 317:1344–1347.

Edgell TC, Lynch BR, Trussell GC, Palmer AR (2009) Experimental evidence for the rapid evolution of behavioral canalization in natural populations. Am Nat 174:434–440.

Endler J. (1977) Geographic Variation, Speciation and Clines. Princeton University Press, Princeton, NJ

Endler JA (1986) Natural Selection in the Wild. Princeton University Press, Princeton

Engelmann J, Hanke W, Mogdans J, Bleckmann H (2000) Hydrodynamic stimuli and the fish lateral line. Nature 408:51–52.

Erickson DL, Fenster CB, Stenøien HK, Price D (2004) Quantitative trait locus analyses and the study of evolutionary process. Mol Ecol 13:2505–2522.

Eronen M, Gluckert G, Hatakka L, van der Plassche O, van der Plicht J, Rantala P (2001) Rates of Holocene isostatic uplift and relative sea-level lowering of the Baltic in SW Finland based on studies of isolation contacts. Boreas 30: 17–30.

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol Bioinform Online 1:47–50.

Fairbairn DJ (1997) Allometry for sexual size dimorphism: patterns and process in the coevolution of body size in males and females. Ann Rev Ecol Syst 28:659–687.

Falconer DS (1952) The problem of environment and selection. Am Nat 86:293–298.

Falconer DS, McKay TFC (1996) Introduction to Quantitative Genetics. Longman, Harlow.

Fidler AE, van Oers K, Drent PJ, Kuhn S, Mueller JC, Kempenaers B (2007) Drd4 gene polymorphisms are associated with personality variation in a passerine bird. Proc R Soc B 274:1685–1691.

Finlay BL, Darlington RB (1995) Linked regularities in the development and evolution of mammalian brains. Science 268:1578–1584.

Finlay BL, Darlington RB, Nicastro N (2001) Developmental structure in brain evolution. BehavBrain Sci 24:263–308.

Fitzpatrick LC (1976) Life history patterns of storage and utilization of lipids for energy in amphibians. Am Zool 16:725-732.

Fitzpatrick JL, Almbro M, Gonzalez-Voyer A, Hamada S, Pennington C, Scanlan C, Kolm N

(2012) Sexual selection uncouples the evolution of brain and body size in pinnipeds. J Evol Biol 25:1321–1330.

Flint J (2003) Analysis of quantitative trait loci that influence animal behaviour. J Neurobiol 54:46-77.

Flint J, Munafo M (2013) Herit-ability. Science 340:1416–1417.

Fong DW, KaneTC, Culver DC (1995) Vestigialization and loss of nonfunctional characters. Ann Rev Ecol Syst 26:249– 268.

Forsman A (1991) Variation in sexual size dimorphism and maximum body size among adder populations: effects of prey size. J Anim Ecol 60:253–267.

Foster JB (1964) Evolution of mammals on islands. Nature 202:234–235.

Foster SA (1999) The geography of behaviour: an evolutionary perspective. Trends Ecol Evol 14:190–195.

Foster SA, Endler JA (1999) Geographic Variation in Behavior: Perspectives on Evolutionary Mechanisms. Oxford University Press, New York

Fowler DC, Liu Y, Ouimet C, Wang Z (2002) The effects of social environment on adult neurogenesis in the female prairie vole. J Neurobiol 51:115–128.

Frommen JG, Herder F, Engqvist L, Mehlis M, Bakker TCM, Schwarzer J, Thünken T (2011) Costly plastic morphological responses to predator specific odour cues in three-spined sticklebacks (*Gasterosteus aculeatus*). Evol Ecol 25:641–656.

Fuiman LA (1993) Development of predator evasion in Atlantic herring, *Clupea harengus* L. Anim Behav 45:1101–1116.

Fuller RC, Houle D (2003) Inheritance of developmental instability. In: Polak M (ed.) Developmental Instability: Causes and Consequences. Oxford: Oxford University Press; pp:157–186

Fuller RC, Carleton KL, Fadool JM, Spady TC, Travis J (2005) Genetic and environmental variation in the visual properties of bluefin killifish, *Lucania goodei*. J Evol Biol 18:516–523.

Fusco G, Minelli A (2010) Phenotypic plasticity in development and evolution: facts and concepts. Phil Trans Roy Soc B 365:547–556.

Gallego A, Heath MR (1994) The development of schooling behaviour in Atlantic herring *Clupea harengus*. J Fish Biol 45:569–588.

Galeotti P, Sacchi R, Vicario V (2005) Fluctuating asymmetry in body traits increases predation risks: tawny owl selection against asymmetric woodmice. Evol Ecol 19:405–418.

Garamszegi LZs, Markó G, Herczeg G (2012) A meta-analysis of correlated behaviours with implications for behavioral syndromes: mean effect size, publication bias, phylogenetic effectsand the role of mediator variables. Evol Ecol 26:1213-1235

Garamszegi LZs, Markó G, Herczeg G (2013) A meta-analysis of correlated behaviours with implications for behavioral syndromes: relationships between particular behavioral traits. Behav Ecol 24:1068-1080

Garvey JE, Ostrand KG, Wahl DH (2004) Energetics, predation, and ration affect sizedependent growth and mortality of fish during winter. Ecology 85:2860–2871.

Ghalambor CK, McKay JK, Carroll SP, Reznick DN (2007) Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. Funct Ecol 21:394–407.

Gibson G (2005) The synthesis and evolution of a supermodel. Science 307:1890-1891.

Gienapp P, Teplitsky C, Alho JS, Mills JA, Merilä J (2008) Climate change and evolution: disentangling environmental and genetic responses. Mol Ecol 17:167-178.

Giles N (1983) The possible role of environmental calcium levels during the evolution of phenotypic diversity in Outer-Hebridean populations of the three-spined

stickleback, *Gasterosteus aculeatus*. J Zool 199:535–544.

Gjedrem T (2000) Genetic improvement of cold-water fish species. Aquacult Res 31:25–33.

Gomulkiewicz R, Kirkpatrick M (1992) Quantitative genetics and the evolution of reaction norms. Evolution 46:390–411.

Gonda A (2011) Intraspecific variation in brain size and architecture: population divergence and phenotypic plasticity. PhD disszertáció, Helsinki Egyetem, Finnország ISBN 978-952-10-7348-9

Gonda A, Herczeg G, Merilä J (2009a) Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? *J Evol Biol* 22: 1721-1726.

Gonda A, Herczeg G, Merilä J (2009b) Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain. Proc R Soc B 276:2085–2092.

Gonda A, Herczeg G, Merilä J (2011) Population variation in brain size of nine-spined sticklebacks (*Pungitius pungitius*) – local adaptation or environmentally induced variation? BMC Evol Biol 11:75.

Gonda A, Herczeg G, Merilä J (2013) Evolutionary ecology of intraspecific brain size variation: a review. Ecol Evol 3:2751-2764.

Gonda A, Välimäki K, Herczeg G, Merilä J (2012) Brain development and predation: plastic responses depend on evolutionary history. Biol Lett 8:249-252.

Gonda A, Trokovic N, Herczeg G, Laurila A, Merilä J (2010) Predation- and competition-mediated brain plasticity in *Rana temporaria* tadpoles. J Evol Biol 23:2300-2308.

Gonzalez-Voyer A, Kolm N (2010) Sex, ecology and the brain: Evolutinary correlates of brain structure volumes in Tanganyikan Cichlids. PLoS One 5:4355.

Gonzalez-Voyer A, Winberg S, Kolm N (2009) Social fishes and single mothers: brain evolution in African cichlids Proc R Soc B 276:161-167.

Gosling SD (2001) From mice to men: what can we learn about personality from animal research. Psychol Bull 127:45–86.

Govardovskii VI, Fyhrquist N, Reuter T, Kuzmin DG, Donner K (2000) In search of the visual pigment template. Vis Neurosci 17:509-528.

Goudet J (2001) FSTAT: A Computer Program to Estimate and Test Gene Diversities and Fixation Indices (version 2.9.3). http://www2.unil.ch/popgen/softwares/fstat.htm

Graham JH, Felley JD (1985) Genomic coadaptation and developmental stability with introgressed populations of *Enneacanthus gloriosus* and *E. obesus.* Evolution 39:72–87.

Green BS (2008) Maternal effects in fish populations. Adv Mar Biol 54: -105.

Green P, Falls K, Crooks S (1990) Documentation for CRI-MAP (version 2.4). Washington University School of Medicine, St. Louis.

Grether GF, Millie DF, Bryant MJ, Reznick DN, Mayea W (2001) Rain forest canopy cover, resource availability, and life history evolution in guppies. Ecology 82:1546–1559.

Gross HP (1979) Geographic variation in European ninespine sticklebacks, *Pungitius pungitius*. Copeia 1979:405-412.

Gyllensten U (1985) The genetic structure of fish: differences in the intraspecific distribution of biochemical genetic variation between marine, anadromous, and freshwater species. J Fish Biol 26:691–699.

Hadfield JD (2010) MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package. J Stat Software 33:1–22.

Hagen DW (1973) Inheritance of number of lateral plates and gill rakers on *Gasterosteus aculeatus*. Heredity 30:303–312.

Haglund TR, Buth DG, Lawson R (1992) Allozyme variation and phylogenetic relationships of Asian, North American, and European populations of the threespine stickleback. Copeia 1992:432-443.

Hall AR, Colegrave N (2008) Decay of unused characters by selection and drift. J Evol Biol 21:610–617.

Hammill E, Rogers A, Beckerman AP (2008) Costs, benefits and the evolution of inducible defences: a case study with *Daphnia pulex*. J Evol Biol 21:705–15.

Harris S, Eroukhmanoff F, Green KK, Svensson EI, Pettersson LB (2011) Changes in behavioural trait integration following rapid ecotype divergence in an aquatic isopod. J Evol Biol 24:1887–1896.

Harvell D (1990) The ecology and evolution of inducible defences. Q Rev Biol 65:323–340.

Heins DC (2012) A research programme in fish ecology leads to a supermodel: a tribute to Professor Robert J. Wooton. Ecol Freshw Fish 21:323-324.

Heins DC, Johnson JM, Baker JA (2003) Reproductive ecology of the nine-spined stickleback from south-central Alaska. J Fish Biol 63:1131-1143.

Heins DC, Baker JA, de Silva G, Birden EL (2005) Clutch characteristics of two populations of nine-spined sticklebacks from south-central Alaska. J Fish Biol 67:873-878.

Henderson ND, Turri MG, DeFries JC, Flint J (2004) QTL analysis of multiple behavioral measures of anxiety in mice. Behav Genet 34:267–293.

Hendry AP, Kelly ML, Kinnison MT, Reznick DN (2006) Parallel evolution of the sexes? Effects of predation and habitat features on the size and shape of wild guppies. J Evol Biol 19:741–754.

Herczeg G, Välimäki K (2011) Intraspecific variation in behaviour: effects of evolutionary history, ontogenetic experience and sex J Evol Biol 24:2434–2444.

Herczeg G, Szabó K, Korsós Z (2005) Asymmetry and population characteristics in dice snakes (*Natrix tessellata*): an interpopulation comparison. Amphibia-Reptilia 26:422-426.

Herczeg G, Gonda A, Merilä J (2009a) Evolution of gigantism in nine-spined sticklebacks. Evolution 63:3190–3200.

Herczeg G, Gonda A, Merilä J (2009b) Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). J Evol Biol 22:544–552.

Herczeg G, Gonda A, Merilä J (2009c). The social cost of shoaling covaries with predation risk in nine-spined stickleback, *Pungitius pungitius*, populations. Anim Behav 77:575–580.

Herczeg G, Turtiainen M, Merilä J (2010a) Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. Biol J Linn Soc 101:413–416.

Herczeg, G., Gonda, A., Merilä, J. (2010b). Rensch's rule inverted – female-driven gigantism in nine-spined stickleback *Pungitius pungitius*. J Anim Ecol 79:581–588.

Herczeg G, Ab Ghani NI, Merilä J (2013) Evolution of stickleback feeding behaviour: genetics of population divergence at different ontogenetic stages. J Evol Biol 26:955-962.

Herczeg G, Gonda A, Kuparinen A, Merilä J (2012) Contrasting growth strategies at high vs. low predation populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? Evol Ecol 26:109–122.

Herczeg G, Välimäki K, Gonda A, Merilä J (2014) Evidence for sex-specific selection in brain: a case study of the nine-spined stickleback. J Evol Biol doi: 10.1111/jeb.12409

Hermida M, Fernández C, Amaro R, San Miguel E (2002) Heritability and "evolvability" of meristic characters in a natural population of *Gasterosteus aculeatus*. Can J Zool 80:532–541.

Hernaman V, Munday PL (2005) Life-history characteristics of coral reef gobies II. Mortality rate, mating system and timing of maturation. Mar Ecol Prog Ser 290:223–237.

Hettinger JA, Liu X, Schwartz CE, Michaelis RC, Holden JJA (2008) A DRD1 haplotype is associated with risk for autism spectrum

disorders in male-only affected sib-pair families. Am J Med Genet Part B 147B:628–636.

Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. Biol J Linn Soc 58:247–276.

Hewitt GM (1999) Post-glacial re-colonization of European biota. Biol J Linn Soc 68:87–112.

Hill WG, Kirkpatrick M (2011) What animal breeding has taught us about evolution. Ann Rev Ecol Evol Syst, 41:1–19.

Hoekstra D, Janssen J (1985) Non-visual feeding behavior of the mottled sculpin, *Cottus bairdi*, in Lake Michigan. Env Biol Fish 12:111–117.

Hohenlohe PA, Bassham S, Etter PD, Stiffler N, Johnson EA, Cresko WA (2010) Population genomics of parallel adaptation in three-spined stickleback using sequenced RAD tags. PLoS Genetics 6, e1000862.

Hoogland R, Morris D, Tinbergen N (1957) The spines of sticklebacks (*Gasterosteus* and *Pygosteus*) as means of defence against predators (*Perca* and *Esox*). Behaviour 10:205–236.

Hosoya S, Kai W, Fujita M, Miyaki K, Suetake H, Suzuki Y, Kikuchi K (2012) The genetic architecture of growth rate in juvenile Takifugu species. Evolution 67:590–598.

Houle D (1992) Comparing evolvability and variability of quantitative traits. Genetics 130:195-204.

Höglund J, Alatalo RV (1995) Leks. Princeton University Press, Princeton, NJ

Huber R, Staaden MJ, van Kaufman LS, Liem KF (1997) Microhabitat use, trophic patterns, and the evolution of brain structure in African cichlids. Brain Behav Evol 50:167–182.

Huntingford FA, Coyle S (2007) Antipredator defenses in sticklebacks: trade-offs, risk sensitivity, and behavioural syndromes. In: Östlund-Nilsson S, Mayer I, Huntingford FA, (eds.) Biology of the three-spined stickleback. CRC Press, Boca Raton, FL. Pp. 127–156

Huntingford FA, Chellappa S, Taylor AC, Strang RHC (2001) Energy reserves and reproductive investment in male three-spined sticklebacks, *Gasterosteus aculeatus*. Ecol Freshw Fish 10:111–117.

Huttunen S, Aspi J (2003) Complex inheritance of male courtship song characters in *Drosophila viridis*. Behav Genet 33:17-24.

Ioannou CC, Krause J (2008) Searching for prey: the effects of group size and number. Anim Behav 75:1383–1388.

Iwaniuk AN, Nelson JE (2002) Can endocranial volume be used as an estimate of brain size in birds? Can J Zool 80:16–23.

Jacobs LF (1996) Sexual selection and the brain. Trends Ecol Evol 11:82–86.

Jerlov NG (1976) Marine Optics. Elsevier, Amsterdam

Johansson F, Andersson J (2009) Scared fish get lazy, and lazy fish get fat. J Anim Ecol 78:772–777.

Jokela-Määttä M, Smura T, Aaltonen A, Ala-Laurila P, Donner K (2007) Visual pigments of Baltic Sea fishes of marine and limnic origin. Vis Neurosci 24:389-398.

Jokela-Määttä M, Vartio A, Paulin L, Donner K (2009) Individual variation in rod absorbance spectra correlated with opsin gene polymorphism in sand goby (*Pomatoschistus minutus*). J Exp Bio 212:3415-3421.

Jones JW, Hynes HBN (1950) The age and growth of *Gasterosteus aculeatus*, *Pygosteus pungitius* and *Spinachia vulgaris*, as shown by their otoliths. J Anim Ecol 19:59-73.

de Jong G (2005) Evolution of phenotypic plasticity: patterns of plasticity and the emergence of ecotypes. New Phytol 166:101–17.

Jönsson KI (1997) Capital and income breeding as alternative tactics of resource use in reproduction. Oikos 78:57–66.

Kalmijn AJ (1988) Hydrodynamic and acoustic field detection. In: Atema J Fay RR, Popper AN, Tavolga WN (eds.) Sensory Biology of Aquatic Animals. Springer-Verlag, Heidelberg, pp 83–130

Kaplan RH (1998) Maternal effects, developmental plasticity and life history evolution. In: Mousseau TA, Fox CW (eds.) Maternal Effects as Adaptations. Oxford University Press, New York, pp 244–260

Karhunen M, Ovaskainen O, Herczeg G, Merilä J (2014) Bringing habitat information into statistical tests of local adaptation in quantitative traits: a case study in nine-spined sticklebacks. Evolution 68:559-568.

Karvonen E, Merilä J, Rintamaki PT, Van Dongen S (2003) Geography of Xuctuating asymmetry in the greenfinch, Carduelis chloris. Oikos 100:507–516.

Kawecki TJ, Ebert D (2004) Conceptual issues in local adaptation. Ecol Lett 7:1225–1241.

Kempermann G, Kuhn HG, Gage FH (1997) More hippocampal neurons in adult mice living in enriched environment. Nature 386:493–495.

Keogh JS, Scott IAW, Hayes C (2005) Rapid and repeated origin of insular gigantism and dwarfism in Australian tiger snake. Evolution 59:226–233.

Kihslinger RL, Nevitt GA (2006) Early rearing environment impacts cerebellar growth in juvenile salmon. J Exp Biol 209:504-509.

Kihslinger RL, Lema SC, Nevitt GA (2006) Environmental rearing conditions produce forebrain differences in wild Chinook salmon *Oncorhynchus tshawytscha*. Comp Biochem Physiol A 145:145-151.

Kingsolver JG, Hoekstra HE, Hoekstra JM, Berrigan D,Vignieri SN, Hill CE, Hoang A, Gibert P, Beerli P (2001) The strength of phenotypic selection in natural populations. Am Nat 157:245-261.

Kitano J, Mori S, Peichel CL (2007) Sexual dimorphism in the external morphology of the threespine stickleback (*Gasterosteus aculeatus*). Copeia 2007:336–349.

Koli L, Rask M, Viljanen M, Aro E (1988) The diet of perch, *Perca fluviatilis* L., at Tvärminne, Northern Baltic Sea, and a comparison with two lakes. Aqua Fenn 18:185–191.

Kolm N, Gonzalez-Voyer A, Brelin D, Winberg S (2009) Evidence for small scale variation in the vertebrate brain: mating strategy and sex affect brain size and structure in wild brown trout (*Salmo trutta*). J Evol Biol 22:2524-2531.

Korsten P, Mueller JC, Hermannstadter C, Bouwman KM, Dingemanse NJ, Drent PJ, Liedvogel M, Matthysen E, van Oers K, van Overveld T, Patrick SC, Quinn JL, Sheldon BC, Tinbergen JM, Kempenaers B (2010) Association between DRD4 gene polymorphism and personality variation in great tits: a test across four wild populations. Mol Ecol 19:832-843.

Kotrschal K, Van Staaden MJ, Huber R (1998) Fish brains: evolution and environmental relationships. Rev Fish Biol Fish 8:373-408.

Kotrschal A, Räsänen K, Kristjansson BK, Senn M, Kolm N (2012) Extreme sexual brain size dimorphism in sticklebacks: a consequence of the cognitive challenges of sex and parenting? PLoS ONE 7: e30055.

Kotrschal A, Rogell B, Bundsen A, Svensson B, Zajitschek S, Brännström I, Immler S, Maklakov AA, Kolm N (2013) Artificial selection on relative brain size in the guppy reveals costs and benefits of evolving larger brains. Curr Biol 23:168–171.

Krause J, Godin J-G J (1995) Predator preferences for attacking particular prey group sizes: consequences for predator hunting success and prey predation risk. Anim Behav 50:465–473.

Krause J, Ruxton GD (2002) Living in Groups. Oxford University Press, Oxford

Kruska D (1988) Mammalian domestication and its effect on brain structure and behavior. In: Jerison HJ, Jerison I (eds.) Intelligence and Evolutionary Biology. Springer-Verlag, Berlin, pp 211-250

Kulczykowska E, Sánchez Vázquez FJ (2010) Neurohormonal regulation of feed intake and response to nutrients in fish: aspects of feeding rhythm and stress. Aquacultural Res 41:654–667.

Kuparinen A, Merilä J (2007) Detecting and managing fisheries-induced evolution. Trends Ecol Evol 22:652–659.

Kuparinen A, Hardie DC, Hutchings JA (2012) Evolutionary and ecological feedbacks of the survival cost of reproduction. Evol Appl 5:245–255.

Laakkonen MVM (2006) The effects of long-term predator exposure on body composition and condition of young Arctic charr (*Salvelinus alpinus*). Ann Zool Fenn 43:263–270.

Lacey EP (1998) What is an environmentally induced parental effect? In: Mousseau TA, Fox CW (eds.) Maternal Effects as Adaptations. Oxford University Press, New York, pp 54–66

Lahti DC, Johnson NA, Ajie BC, Otto SP, Hendry AP, Blumstein DT, Coss RG, Donohue K, Foster SA (2009) Relaxed selection in the wild. Trends Ecol Evol 24:487–496.

Laine VN, Herczeg G, Shikano T, Primmer CR (2012a) Heterozygosity–behaviour correlations in nine-spined stickleback (*Pungitius pungitius*) populations: contrasting effects at random and functional loci. Mol Ecol 21:4872-4884.

Laine VN, Primmer CR, Herczeg G, Merilä J, Shikano T (2012b) Isolation and characterization of 13 new nine-spined stickleback, *Pungitius pungitius*, microsatellites located nearby candidate genes for behavioural variation. Ann Zool Fenn 49:123–128.

Laine VN, Shikano T, Herczeg G, Vilkki J, Merilä J (2013) Quantitative trait loci for growth and body size in the nine-spined stickleback *Pungitius pungitius* L. Mol Ecol 22:6861-5876.

Laine VN, Herczeg G, Shikano T, Vilkki J, Merilä J (2014) QTL analysis of behavior in nine-spined sticklebacks (*Pungitius pungitius*). Behav Genet 44:77-88.

Laming PR, McKinley SJ (1990) Habituation in goldfish (Carassius auratus) is impaired by increased interstimulus interval, interval variability, and telencephalic ablation. Behav Neurosci 104: 869–875.

Lande R (1976) Natural selection and random genetic drift in phenotypic evolution. Evolution 30: 314-334.

Larmuseau MH, Raeymaekers JA, Ruddick KG, Van Houdt JK, Volckaert FA (2009) To see in different seas: spatial variation in the rhodopsin gene of the sand goby (*Pomatoschistus minutus*). Mol Ecol 18:4227-4239.

Larmuseau MH, Vancampenhout K, Raeymaekers JA, Van Houdt JK, Volckaert FA (2010) Differential modes of selection on the rhodopsin gene in coastal Baltic and North Sea populations of the sand goby, *Pomatoschistus minutus*. Mol Ecol 19:2256-2268.

Laugen AT, Laurila A, Merilä J (2002) Maternal and genetic contributions to geographical variation in *Rana temporaria* larval life-history traits. Biol J Linn Soc 76:61–70.

Laurila A, Karttunen S, Merilä J (2002) Adaptive phenotypic plasticity and genetics of larval lifehistories in two *Rana temporaria* populations.Evolution 56:617–627.

Leamy LJ (1997) Is developmental stability heritable? J Evol Biol 10:21–29.

Leary RF, Allendorf FW (1989) Fluctuating asymmetry as an indicator of stress: implications for conservation biology. Trends Ecol Evol 4:214–217.

Leinonen T, Cano Arias JM, Mäkinen H, Merilä J (2006) Contrasting patterns of body shape and neutral genetic divergence in marine and lake populations of threespined sticklebacks. J Evol Biol 19:1803–1812.

Leinonen T, O'Hara RB, Cano JM; Merilä J (2008) Comparative studies of quantitative trait and neutral marker divergence: a meta-analysis. J Evol Biol 21:1-17.

Leinonen T, Cano JM, Merilä J (2010) Genetics of body shape and armour variation in threespine sticklebacks. J Evol Biol 24:206-218.

Leinonen T, Cano JM, Merilä J (2011) Genetic basis of sexual dimorphism in the threespine stickleback *Gasterosteus aculeatus*. Heredity 106:218-227.

Leinonen T, Herczeg G, Cano JM, Merilä J (2011) Predation-imposed selection on threespine stickleback (*Gasterosteus aculeatus*) morphology: a test of the refuge use hypothesis. Evolution 65:2916-2926.

Leinonen T, McCairns RJ, O'Hara RB, Merilä J (2013) *Q*ST–*F*ST comparisons: evolutionary and ecological insights from genomic heterogeneity. Nat Rev Genet 14:179-190.

Lema SC, Hodges MJ, Marchetti MP, Nevitt GA (2005) Proliferation zones in the salmon telencephalon and evidence for environmental influence on proliferation rate. Comp Biochem Physiol A 141:327–335.

Lens L, van Dongen S, Kark S, Matthysen E (2002) Fluctuating asymmetry as an indicator of stress: can we bridge the gap between studies? Biol Rev 77:27–38.

Lens L, Van Dongen S, Kark S, Talloen W, Hens L, Matthysen E (2001) The use of bilateral asymmetry in ecology and conservation: concept, developments and prospects. Recent Res Dev Ecol 1:21–41.

Leung B, Forbes MR (1996) Fluctuating asymmetry in relation to stress and fitness: effects of trait type as revealed by meta-analysis. Ecoscience 3:400–413.

Leung B, Forbes MR, Houle D (2000) Fluctuating asymmetry as a bioindicator of stress: comparing efficacy of analyses involving multiple traits. Am Nat 155:101–115.

Lima SL, Dill LM (1990) Behavioural decisions made under the risk of predation- a review and prospectus. Can J Zool 68:619–640.

Lind MI, Ingvarsson PK, Johanson H, Hall D, Johansson F (2011) Gene flow and selection on phenotypic plasticity in an island system of *Rana temporaria*. Evolution 65:684–697.

Lipkind D, Nottebohm R, Rado R, Barnea A (2002) Social change affects the survival of new neurons in the forebrain of adult songbirds. Behav Brain Res 133:31–43.

Lisney TJ, Bennett MB, Collin SP (2007) Volumetric analysis of brain areas indicates a shift in the relative importance sensory system in elasmobranchs. Raffl Bull Zool 14:7-15.

Lomolino MV (1985) Body size of mammals on islands: the island rule re-examined. Am Nat 125:310–316.

Lomolino MV (2005) Body size evolution in insular vertebrates: generality of the island rule. J Biogeogr 32:1683–1699.

Lugo JM, Rodriguez A, Helguera Y, Morales R, Gonzales O,Acosta J, Besada V, Sanchez A, Estrada MP (2008) Recombinant novel pituitary adenylate cyclase-activating polypeptide from African catfish (*Clarias gariepinus*) authenticates its biological function as a growth-promoting factor in low vertebrates. J Endocrin 197:583–597.

Lynch M, Walsh JB (1998) Genetics and Analysis of Quantitative Traits. Sinauer Associates. Sunderland, MA

Lynch M, Pfrender M, Spitze K, Lehman N, Hicks J, Allen D, Latta L, Ottene M, Bogue F, Colbourne J (1999) The quantitative and molecular genetic architecture of a subdivided species. Evolution 53:100–110.

Mackay TFC, Stone EA, Ayroles JF (2009) The genetics of quantitative traits: challenges and prospects. Nat Rev Genet 10:565–577.

Madsen T, Shine R (1993) Phenotypic plasticity in body sizes and sexual size dimorphism in European grass snakes. Evolution 47:321–325.

Maguire EA, Gadian DG, Johnsrude IS, Good CD, Ashburner J, Frackowiak RSJ, Frith CD (2000) Navigation-related structural change in the hippocampi of taxi drivers. Proc Natl Acad Sci USA 97:4397-4403.

Magurran AE (1990a) The adaptive significance of schooling as an anti-predator defence in fish. Ann Zool Fenn 27:51–66.

Magurran AE (1990b) The inheritance and development of minnow anti-predator behaviour. Anim Behav 39:834–842.

Magurran AE (1999) The diversification and development of antipredator behaviour: perspectives from fish populations. In: Foster

SA, Endler JA (eds.) Geographic Diversification of Behavior: an Evolutionary Perspective. Oxford University Press, Oxford pp 139–163

Magurran A E, Pitcher TJ (1983) Foraging, timidity and shoal size in minnows and goldfish. Behav Ecol Sociobiol 12:142–152.

Magurran AE, Seghers BH (1991) Variation in schooling and aggression amongst guppy (*Poecilia reticulata*) populations in Trinidad. Behaviour 118:214–234.

Magurran AE, Seghers BH (1994) Predator inspection behaviour covaries with schooling tendency amongst wild guppy, *Poecilia reticulata*. Behaviour 128:121–134.

Mangel M, Munch SB (2005) A life-history perspective of short- and long-term consequences of compensatory growth. Am Nat 166:E155–E176.

Marchinko KB (2009) Predation's role in repeated phenotypic and genetic divergence of armor in threespine stickleback. Evolution 63:127–138.

Marchinko KB, Schluter D (2007) Parallel evolution by correlated response: lateral plate reduction in threespine stickleback. Evolution 61:1084–1090.

Martín J, López P (2000) Chemoreception, symmetry and mate choice in lizards. Proc Roy Soc Lond B 267:1265–1269.

Martín J, López P (2001) Hindlimb asymmetry reduces escape performance in the lizard Psammodromus algirus. Physiol Biochem Zool 74:619–624.

Masel J, King OD, Maughan H (2007) The loss of adaptive plasticity during long periods of environmental stasis. Am Nat 169:38–46.

Mayr E (1963) Animal species and evolution. Harvard University Press, Cambridge

Mayr E (1967) The challenge of island faunas. Austr Nat Hist 15:369–374.

McGuigan K, Chenoweth SF, Blows MW (2005) Phenotypic divergence along lines of genetic variance. Am Nat 165:32–43.

McHenry M J, Feitl KE, Strother JA, Van Trump WJ (2009) Larval zebrafish rapidly sense the water flow of a predator's strike. Biol Lett 5:477–479.

McNamara JM, Houston AI (1990) The value of fat reserves and the tradeoff between starvation and predation. Acta Biotheor 38:37–61.

McPhail JD (1963) Geographic variation in North American ninespine sticklebacks, *Pungitius pungitius*. J Fish Res Board Can 20:27-44.

de Meester L (1993) Fish-mediated chemical, and phototactic behaviour in *Daphnia magna*. Ecology 74:1467–1474.

de Meester L (1996) Evolutionary potential and local genetic differentiation in a phonetically plastic trait of a cyclical parthenogen, *Daphnia magna*. Evolution 50:1293–1298.

Merilä J (1997) Quantitative trait and allozyme divergence in the greenfinch (*Carduelis chloris*, Aves: Fringillidae). Biol J Linn Soc 61:243–266.

Merilä J (2009) It's the genotype, stupid! J Anim Breed Genet 126:1–2.

Merilä J (2013) Nine-spined stickleback (*Pungitius pungitius*): an emerging model for evolutionary biology research. Ann N Y Acad Sci 1289:18-35.

Merilä J, Björklund M (1995) Fluctuating asymmetry and measurement error. Syst Biol 44:97–101.

Merilä J, Crnokrak P (2001) Comparison of genetic differentiation at marker loci and quantitative traits. J Evol Biol 14:892–903.

Merilä J, Björklund M, Baker AJ (1997) Historical demography and present day population structure of the greenfinch, *Carduelis chloris*—an analysis of mtDNA control-region sequences. Evolution 51:946–956.

Merry JW, Kemp DJ, Rutowski RL (2011) Variation in compound eye structure: effects of diet and family. Evolution 65: 2098–2110.

Messler A, Wund MA, Baker JA, Foster SA (2007) The effects of relaxed and reversed

selection by predators on the antipredator behavior of the threespine stickleback, *Gasterosteus aculeatus*. Ethology 113:953–963.

Michel C, Hicks BJ, Stölting KN, Clarke AC, Stevens MI, Tana R, Meyer A, van den Heuvel MR (2008) Distinct migratory and nonmigratory ecotypes of an endemic New Zealand eleotrid (*Gobiomorphus cotidianus*) – implications for incipient speciation in island freshwater fish species. BMC Evol Biol 8:49.

Miller CT, Beleza S, Pollen AA, Schluter D, Kittles RA, Shriver RMD, Kingsley DM (2007) Cis-regulatory changes in kit ligand expression and parallel evolution of pigmentation in sticklebacks and humans. Cell 131:1179–1189.

Mobley KB, Lussetti D, Johansson F, Englund G, Bokma F (2011) Morphological and genetic divergence in Swedish postglacial stickleback (*Pungitius pungitius*) populations. BMC Evol Biol 11:287.

Mohammed AH, Zhu SW, Darmopil S, Hjerling-Leffler J, Ernfors P, Winblad B, Diamond MC, Eriksson PS, Bogdanovic N (2002) Environmental enrichment and the brain. Prog Brain Res 138:109–133.

Moodie GEE (1972a) Predation, natural selection and adaptation in an unusual threespine stickleback. Heredity 28:155–167.

Moodie GEE (1972b) Morphology, life history and ecology of an unusual stickleback (*Gasterosteus aculeatus*) in the Queen Charlotte Islands. Can J Zool 50:721–738.

Moodie GEE, Reimchen TE (1976) Phenetic variation and habitat differences in *Gasterosteus* populations of the Queen Charlotte Islands. Syst Zool 25:49–61.

Møller AP (2010) Brain size, head size and behaviour of a passerine bird. J Evol Biol 23:625-635.

Montgomery JC, Macdonald JA (1987) Sensory tuning of lateral line receptors in Antarctic fish to the movements of planktonic prey. Science 235:195–196.

Montgomery JC, Milton RC (1993) Use of the lateral line for feeding in the torrentfish (Cheimarrichthys fosteri). New Zealand J Zool 20:121–125.

Montgomery J, Coombs S, Halstead M (1995) Biology of the mechanosensory lateral line in fishes. Rev Fish Biol Fish 5:399–416.

Montgomery JC, Baker CF, Carton AG (1997) The lateral line can mediate rheotaxis in fish. Nature 389:960–963.

Montgomery JC, Coombs S, Baker CF (2001) The mechanosensory lateral line system of the hypogean form of *Astyanax fasciatus*. Environ Biol Fish 62:87–96.

Moran N (1992) The evolutionary maintenance of alternative phenotypes. Am Nat 139:971–989.

Moran P, Izquierdo JI, Pendas AM, GarciaVazquez E (1997) Fluctuating asymmetry and isozyme variation in Atlantic salmon: relation to age of wild and hatchery fish. Trans Am Fish Soc 126:194–199.

Mousseau TA, Fox CW (1998) Maternal Effects as Adaptations. Oxford University Press, New York

Van Netten S, Kroese ABA (1987) Laser interferometric measurements on the dynamic behavior of the cupula in the fish lateral line. Hearing Res 29:55–62.

Ocalewicz K, Fopp-Bayat D, Woznicki P, Jankun M (2008) Heteromorphic sex chromosomes in the ninespine stickleback *Pungitius pungitius*. J Fish Biol 73:456–462.

Van Oers K, Mueller JC (2010) Evolutionary genomics of animal personality. Phyl Trans R Soc B 365:3991–4000.

van Oers K, de Jong G, van Noordwijk AJ, Kempenaers B, Drent PJ (2005) Contribution of genetics to the study of animal personalities: a review of case studies. Behaviour 142:1191–1212.

O'Hara RB, Merilä J (2005) Bias and precision in Qst estimates: problems and some solutions. Genetics 171:1331-1339.

Opstad R, Rogers SM, Behmer ST, Simpson SJ (2004) Behavioural correlates of phenotypic

plasticity in mouthpart chemoreceptor numbers in locusts. J Ins Physiol 50:725–736.

Ortí G, Bell MA, Reimchen TE, Meyer A (1994) Global survey of mitochondrial-DNA sequences in the threespine stickleback: evidence for recent migrations. Evolution 48:608-622.

Östlund-Nilsson S, Mayer I, Huntingford FA (2007) Biology of the Three-Spined Stickleback. CRC Press, Boca Raton

Palmer AR (1999) Detecting publication bias in meta-analyses: a case study in of fluctuating asymmetry and sexual selection. Am Nat 154:220–233.

Palmer AR, Strobeck C (1986) Fluctuating asymmetry: measurement, analysis, patterns. Ann Rev Ecol Syst 17:391–421.

Palo JU, Schmeller D, Laurila A, Primmer CR, Kuzmin Sl, Merilä J (2004) High degree of population subdivision in a widespread amphibian. Mol Ecol 13:2631–2644.

Parsons PA (1992) Fluctuating asymmetry: a biological monitor of environmental and genomic stress. Heredity 68:361–364.

Partridge BL, Pitcher TJ (1980) The sensory basis of fish schools, relative roles of lateral line and vision. J Comp Physiol 135:315–325.

Pauwels K, Stoks R, Meester L De (2010) Enhanced antipredatory defence in the presence of food stress in the water flea *Daphnia magna*. Funct Ecol 24:322–329.

Peichel CL, Nereng KS, Ohgi KA, Cole BLE, Colosimo PF, Buerkle CA, Schluter D, Kingsley DM (2001) The genetic architecture of divergence between threespine stickleback species. Nature 414:901-905.

Pelletier D, Dutil J, Blier P, Guderley H (1994) Relation between growth rate and metabolic organization of white muscle , liver and digestive tract in cod , *Gadus morhua*. J Comp Physiol B 164:179–190.

Perez-Barberia FJ, Shultz S, Dunbar RIM (2007) Evidence for coevolution of sociality and relative brain size in three orders of mammals. Evolution 61:2811–2821.

Pérez-Tris J, Díaz JA, Tellería JL (2004) Loss of body mass under predation risk: cost of antipredatory behaviour or adaptive fit-forescape? Anim Behav 67:511–521.

Peters RH (1983) The ecological implications of body size. Cambridge University Press, Cambridge

Pfennig DW, Wund MA, Snell-Rood EC, Cruickshank T, Schlichting CD, Moczek AP (2010) Phenotypic plasticity's impacts on diversification and speciation. Trends Ecol Evol 25:459–467.

Pigliucci M (2005) Evolution of phenotypic plasticity: where are we going now? Trends Ecol Evol 20:481–486.

Pigliucci M, Murren CJ, Schlichting CD (2006) Phenotypic plasticity and evolution by genetic assimilation. J Exp Biol 209:2362–2367.

Pitcher TJ, Parrish JK (1993) Functions of shoaling behaviour in teleosts. In: Pitcher TJ (ed.) Behaviour of Teleost Fishes, 2nd edn. Chapman & Hall, London, pp 363–439

Pitcher TJ, Partridge BL, Wardle CS (1976) A blind fish can school. Science 194: 963–965.

Pitcher TJ, Magurran AE, Winfield IJ (1982) Fish in larger shoals find food faster. Behav Ecol Sociobiol 10:149–151.

Podisi BK, Knott SA, Burt DW, Hocking PM (2013) Comparative analysis of quantitative trait loci for body weight, growth rate and growth curve parameters from 3 to 72 weeks of age in female chickens of a broiler-layer cross. BMC Genetics 14:22.

Polak M, Taylor PW (2007) A primary role of developmental instability in sexual selection. Proc R Soc B 274:3133–3140.

Pollen AA, Dobberfuhl AP, Scace J, Igulu MM, Renn SCP, Shumway CA, Hofmann HA (2007) Environmental complexity and social organization sculpt the brain in Lake Tanganyikan cichlid fish. Brain Behav Evol 70:21-39.

Portavella M, Salas C, Vargas JP, Papini MR (2003) Involvement of telencephalon in spaced-

trial avoidance learning in the goldfish (*Carassius auratus*). Phys Behav 80:49–56.

Poulin R (1999) Parasitism and shoal size in juvenile sticklebacks: conflicting selection pressures from different ectoparasites. Ethology 105:959–968.

Poulin R, Fitzgerald GJ (1989) Shoaling as an anti-ectoparasite mechanism in juvenile sticklebacks (Gasterosteus ssp.). Behav Ecol Sociobiol 24:251–255.

Van Praag H, Kempermann G, Cage FH (2000) Neural consequences of environmental enrichment. Nat Rev 1:191–198.

Pratt TC, Fox MG (2002) Influence of predation risk on the overwinter mortality and energetic relationships of young-of-year Walleyes. Trans Am Fish Soc131:885–898.

Pravosudov VV, Kitaysky AS, Omanska A (2006) The relationship between migratory behaviour, memory and the hippocampus: an intraspecific comparison. Proc R Soc B 273:2641-2649.

Pritchard JR, Schluter D (2001) Declining interspecific competition during character displacement: summoning the ghost of competition past. Evol Ecol Res 3:209–220.

Pujol B, Wilson AJ, Ross RIC Pannell JR (2008) Are QST - FST comparisons for natural populations meaningful? Mol Ecol 17:4782–4785.

Quinn TP, Unwin MJ, Kinnison MT (2000) Evolution of temporal isolation in the wild: genetic divergence in timing of migration and breeding by introduced Chinook salmon populations. Evolution 54:1372–1385.

R Development Core Team 2007. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Reale D, Reader SM, Sol D, McDougall PT, Dingemanse NJ (2007) Integrating animal temperament within ecology and evolution. Biol Rev 82:291–318.

Reif A, Lesch K-P (2003) Toward a molecular architecture of personality. Behav Brain Res 139:1–20.

Reimchen TE (1983) Structural relationships between spines and lateral plates in threespine stickleback (*Gasterosteus aculeatus*). Evolution 37:931–946.

Reimchen TE (1988) Inefficient predators and prey injuries in a population of giant stickleback. Can J Zool 66:2036–2044.

Reimchen TE (1991) Trout foraging failure and the evolution of body size in stickleback. Copeia 1991:1098–1104.

Reimchen TE (1992) Injuries on stickleback from attacks by a toothed predator (*Oncorhynchus*) and implications for the evolution of lateral plates. Evolution 46:1224–1230.

Reimchen TE (1994) Predators and morphological evolution in threespine stickleback. In: Bell MA, Foster SA (eds.) The evolutionary biology of the threespine stickleback. Oxford University Press, Oxford, UK. pp 240–276

Reimchen TE, Stinson EM, Nelson JS (1985) Multivariate differentiation of parapatric and allopatric populations of threespine stickleback in the Sangan River watershed, Queen Charlotte Islands. Can J Zool 63:2944–2951.

Rensch B (1950) Die Abhängigkeit der relativen Sexualdifferenz von der Körpergrösse. Bonn Zool Beitr 1:58–69.

Rensch B (1959) Evolution above Species Level. Columbia University Press, New York

Ressler KJ, Mercer KB, Bradley B, Jovanovic T, Mahan A, Kerley K, Norrholm SD, Kilaru V, Smith AK, Myers AJ, Ramirez M, Engel A, Hamamck SE, Toufexis D, Braas KM, Binder EB, May V (2011) Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. Nature 470:492–497.

Reznick D (1982) The impact of predation on life history evolution in Trinidadian guppies: genetic basis of observed life history patterns. Evolution 36:1236–1250.

Reznick D, Endler JA (1982) The impact of predation on life history evolution in Trinidadian guppies (*Poecilia reticulata*). Evolution 36:160–177.

Reznick DN, Ghalambor CK (2005) Can commercial fishing cause evolution? Answers from guppies (*Poecilia reticulata*). Can J Fish Aquat Sci 62:791–801.

Reznick D, Bryga H, Endler JA (1990) Experimentally induced life-history evolution in a natural population. Nature 346:357–359.

Reznick DN, Butler IV MJ, Rodd FH, Ross P (1996) Life-history evolution in guppies (*Poecilia reticulata*): 6. Differential mortality as a mechanism for natural selection. Evolution 50:1651–1660.

Reuter TE, White RH, Wald G (1971) Rhodopsin and porphyropsin fieldsin the adult bullfrog retina. J Gen Physiol 58:351-371.

Riska B, Atchley WR, Rutledge JJ (1984) A genetic analysis of targeted growth in mice. Genetics 107:79–101.

Robards MD, Anthony JA, Rose GA, Piatt JF (1999) Changes in proximate composition and somatic energy content for Pacific sand lance (*Ammodytes hexapterus*) from Kachemak Bay, Alaska relative to maturity and season. J Exp Mar Biol Ecol242:245–258.

Rocha JL, Eisen EJ, Dale Van Vleck L, Pomp D (2004) A large-sample QTL study in mice: I Growth. Mamm Genome 83–99.

Rodd FH, Sokolowski MB (1995) Complex origins of variation in the sexual behaviour of male Trinidadian guppies, *Poecilia reticulata*: interactions between social environment, heredity, body size and age. Anim Behav 49:1139–1159.

Roff DA (1992) The evolution of life histories. Chapmann & Hall, New York

Roff DA (2002) Life History Evolution. Sinauer Associates, Sunderland, MA

Rogell B, Dannewitz J, Palm S, Petersson E, Dahl J, Prestegaard T, Järvi T, Laurila A (2012) Strong divergence in trait means but not in plasticity across hatchery and wild populations of sea-run brown trout *Salmo trutta*. Mol Ecol 21:2963–2976.

Rohlf FJ (2006) *TPS software series*. Distributed by the author. Stony Brook, NY: Department of Ecology and Evolution, State University of New York.

Roldan RS, Cassinello T, Abaigar T, Gomeindo M (1998) Inbreeding, fluctuating asymmetry and ejaculate quality in an endangered ungulate. Proc R Soc Lond B 265:243–248.

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.

Rossiter M (1996) Incidence and consequences of inherited environmental effects. Ann Rev Ecol Syst 27:451–476.

Rossiter M (1998) The role of environmental variation in parental effects expression. In: Mousseau TA, Fox CW (eds.) Maternal Effects as Adaptations. Oxford University Press, New York, pp 112–134

Roth TC, Pravosudov VV (2009) Hippocampal volumes and neuron numbers increase along a gradient of environmental harshness: a large-scale comparison. Proc R Soc B 276:401-406.

Saarinen PK, Pahlberg J, Herczeg G, Viljanen M, Karjalainen M, Shikano T, Merilä J, Donner K (2012) Spectral tuning by selective chromophore uptake in rods and cones of eight populations of nine-spined stickleback (*Pungitius pungitius*). J Exp Biol 215:2760–2773.

Salonen A, Peuhkuri N (2007) Aggression level in different water velocities depends on population origin in grayling, *Thymallus thymallus*. Ethology 113:39–45.

Sarre S (1996) Habitat fragmentation promotes fluctuating asymmetry but not morphological divergence in two geckos. Res Pop Ecol 38:57–64.

Schlötterer C (2003) Hitchhiking mapping – functional genomics from population genetic perspective. Trends Genet 19:32-38.

Schluter D (2000) The ecology of adaptive radiation. Oxford University Press, Oxford

Schluter D, Nagel LM (1995) Parallel speciation by natural selection. Am Nat 146:292–301.

Schultz ET, Conover DO (1997) Latitudinal differences in somatic energy storage: adaptive responses to seasonality in an estuarine fish (Atherinidae: *Menidia menidia*). Oecologia 109:516–529.

Schütz KE, Kerje S, Jacobsson L, Forkman B, Carlborg Ö, Andersson L, Jenssen P (2004) Major growth QTLs in fowl are related to fearful behavior: possible genetic links between fear responses and production traits in a red junglefowl x white leghorn intercross. Behav Gen 34:121–130.

Seaton G, Hernandez J, Grunchec J, White I, Allen J, De Koning DJ, Wei W, Berry D, Haley C, Knott S (2006) GridQTL: a grid portal for QTL mapping of compute intensive datasets. Proceedings of the 8th World Congress on Genetics Applied to Livestock. Belo Horizonte, Brazil.

Serrano-Meneses MA, Cordoba-Aguilar A, Azpilicueta-Amorin M, Gonzalez-Soriano E, Székely T (2009) Sexual selection, sexual size dimorphism and Rensch's rule in Odonata. J Evol Biol 21:1259–1273.

Shapiro MD, Marks ME, Peichel CL, Blackman BK, Nereng KS, Jónsson B, Schluter D, Kingsley DM (2004) Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature 428:717-723.

Shapiro MD, Bell MA, Kingsley DM (2006) Parallel genetic origins of pelvic reduction in vertebrates. Proc Natl Acad Sci USA 103:13753–13758.

Shapiro MD, Summers BR, Balabhadra S, Miller AL, Cunningham CB, Aldenhoven JT, Bell MA, Kingsley DM (2009) The genetic architecture of skeletal convergence and sex determination in ninespine sticklebacks. Curr Biol 19:1140–1145.

Shikano T, Ramadevi J, Shimada Y, Merilä J (2010a) Utility of sequenced genomes for microsatellite marker development in non-model organisms: a case study of functionally important genes in nine-spined sticklebacks (*Pungitius pungitius*). BMC Genomics 11: 334.

Shikano T, Ramadevi J, Merilä J (2010b) Identification of local- and habitat-dependent selection: Scanning functionally important genes in nine-spined sticklebacks (*Pungitius pungitius*). Mol Biol Evol 27:2775-2789.

Shikano T, Shimada Y, Herczeg G, Merilä J (2010c) History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (Pungitius pungitius) populations. Mol Ecol 19:1147–1161.

Shikano T, Laine VN, Herczeg G, Vilkki J, Merilä J (2013) Genetic architecture of parallel pelvic reduction in ninespine sticklebacks. G3:Genes, Genomes, Genetics 3:1833-1842.

Shimada Y, Shikano T, Merilä J (2011b) A high incidence of selection on physiologically important genes in the three-spined stickleback, *Gasterosteus aculeatus*. Mol Biol Evol 28:181–193.

Shine R (1989) Ecological causes for the evolution of sexual size dimorphism: a review of the evidence. Q Rev Biol 64:419–461.

Shirvell CS (1981) Validity of fin-ray ageing for brown trout. J Fish Biol 18:377–383.

Sibly R, Calow P (1986) Why breeding earlier is always worthwhile. J Theor Biol 124:311–319.

Sih A, Bell A, Johnson JC (2004a) Behavioural syndromes: an ecological and evolutionary overview. Trends Ecol Evol 19:372–378.

Sih A, Bell AM, Johnson JC, Ziemba RE (2004b) Behavioral syndromes: an integrative overview. Q. Rev. Biol. 79: 241–277.

Siikamäki P, Lammi A (1998) Fluctuating asymmetry in central and marginal populations of *Lychnis viscaria* in relation to genetic and environmental factors. Evolution 52:1285–1292.

Simberloff D, Dayan T, Jones C, Ogura G (2000) Character displacement and release in the small Indian mongoose, *Herpestes javanicus*. Ecology 81:2086–2099.

Slate J (2005) Quantitative trait locus mapping in natural populations: progress, caveats and future directions. Mol Ecol 14:363–379.

Smith AR, D'Annunzio L, Smith AE, Sharma A, Hofmann CM, Marshall NJ, Carleton K L (2011) Intraspecific cone opsin expression variation in the cichlids of Lake Malawi. Mol Ecol 20:299–310.

Sogard SM (1997) Size selective mortality in the juvenile stages of teleost fishes: a review. Bull Mar Sci 60:1129–1157.

Sogard SM, Spencer ML (2004) Energy allocation in juvenile sablefish: effects of temperature , ration and body size. J Fish Biol 64:726–738.

Spicer JI, Gaston KJ (1999) Physiological Diversity and its Ecological Implications. Blackwell Science, Oxford

Spitze K (1993) Population structure in *Daphnia obtusa*: quantitative genetic and allozymic variation. Genetics 135:367-374.

Van Staaden M, Huber R, Kaufman L, Liem K (1995) Brain evolution in cichlids of the African Great Lakes: brain and body size, general patterns and evolutionary trends. Zoology 98:165–178.

Stahlschmidt ZR, Davis JR, Denardo DF (2011) Sexual variation in assimilation efficiency: its link to phenotype and potential role in sexual dimorphism. J Comp Physiol B 181:383–389.

Stearns SC (1983) The genetic basis of differences in life-history traits among six populations of mosquitofish (*Gambusia affinis*) that shared ancestors in 1905. Evolution 37:618–627.

Stearns SC (1984) Heritability estimates for age and length at maturity in two populations of mosquito fish that shared ancestors in 1905. Evolution 38:368–375.

Stearns SC (1992) The evolution of life histories. Oxford University Press, Oxford, U.K.

Storz JF (2005) Using genome scans of DNA polymorphism to infer adaptive population divergence. Mol Ecol 14:671-688.

Striedter GF (2005) Principles of Brain Evolution. Sinauer Associates. Sunderland

Swaddle JP (1997) Developmental stability and predation success in an insect predator-prey system. Behav Ecol 8:433–436.

Sultan SE, Spencer HG (2002) Metapopulation structure favors plasticity over local adaptation. Am Nat 160:271–283.

Székely T, Freckleton RB, Reynolds JD (2004) Sexual selection explains Rensch's rule of size dimorphism in shorebirds. Proc Natl Acad Sci USA 101:12224–12227.

Takahashi H, Goto A (2001) Evolution of East Asian ninespine sticklebacks as shown by mitochondrial DNA control region sequences. Mol Phylogenet Evol 21:135–155.

Takata K, Goto A, Yamazaki F (1987) Biochemical identification of a brackish water type of *Pungitius pungitius*, and its morphological and ecological features in Hokkaido, Japan. Jap J Ichth 34:176–183.

Teacher AGF, Shikano T, Karjalainen ME, Merilä J (2011) Phylogeography and Genetic Structuring of European Nine-Spined Sticklebacks (*Pungitius pungitius*)—Mitochondrial DNA Evidence. PLoS ONE 6(5): e19476.

Temple SE, Plate EM, Ramsden S, Haimberger TJ, Roth WM, Hawryshyn CW (2006) Seasonal cycle in vitamin A1/A2-based visual pigment composition during the life history of coho salmon (*Oncorhynchus kisutch*). J Comp Physiol A 192:301-313.

Teplitsky C, Plénet S, Joly P (2005) Costs and limits of dosage response to predation risk: to what extent can tadpoles invest in anti-predator morphology? Oecologia 145:364–370.

Terai Y, Seehausen O, Sasaki T, Takahashi K, Mizoiri S, Sugawara T, Sato T, Watanabe M, Konijnendijk N, Mrosso HD, Tachida H, Imai H, Shichida Y, Okada N (2006) Divergent selection on opsins drives incipient speciation in Lake Victoria cichlids. PLoS Biol 4:e433.

Tollrian R (1995) Predator-induced morphological defenses: costs, life history shifts,

and maternal effects in *Daphnia pulex*. Ecology 76:1691–1705.

Tollrian R, Harvell CD (1999) The ecology and evolution of inducible defenses. Princeton University Press, Princeton, NJ

Torres-Dowdall J, Handelsman CA, Reznick DN, Ghalambor CK (2012) Local adaptation and the evolution of phenotypic plasticity in Trinidadian guppies (*Poecilia reticulata*). Evolution 66:3432-3443.

Tramontin AD, Brenowitz EA (2000) Seasonal plasticity in adult brain. Trends Neurosci 23:251-258.

Trokovic N, Herczeg G, McCairns RJS, Ab Ghani NI, Merilä J (2011a) Intraspecific divergence in the lateral line system in the nine-spined stickleback (*Pungitius pungitius*). J Evol Biol 24:1546-1558.

Trokovic N, Gonda A, Herczeg G, Laurila A, Merilä J (2011b) Brain plasticity over the metamorphic boundary: carry-over effect of larval environment on froglet brain development. J Evol Biol 24:1380-1385.

Trokovic N, Herczeg G, Ab Ghani NI, Shikano T, Merilä J (2012) High levels of fluctuating asymmetry in isolated stickleback populations. BMC Evol Biol 12:115.

Urban MC (2007) The growth-predation risk trade-off under a growing gape-limited predation threat. Ecology 88:2587–2597.

Urban MC (2008) Salamander evolution across a latitudinal cline in gapelimited predation risk. Oikos 117:1037–1049.

Van Valen L (1962) A study of fluctuating asymmetry. Evolution 16:125–142.

Van Valen LM (1973a) Body size and the number of plants and animals. Evolution 27:27–35.

Van Valen LM (1973b) Pattern and the balance of nature. Evol Theor 1:31–49.

Välimäki K (2012) Intraspecific variation in phenotypic plasticity. PhD disszertáció, Helsinki Egyetem, Finnország ISBN 978-952-10-8427-0

Välimäki K, Herczeg G (2012) Ontogenetic and evolutionary offects of predation and competition on nine-spined stickleback (*Pungitius pungitius*) body size. J Anim Ecol 81:859-867.

Välimäki K, Herczeg G, Merilä J (2012) Morphological antipredator defences in the nine-spined sticklebacks: constitutive, induced or both? Biol J Linn Soc 107:854-866.

Våge J, Wade C, Biagi T, Fatjó J, Amat M, Lindblad-Toh K, Lingaas F (2010) Association of dopamine- and serotonin-related genes with canine aggression. Genes Brain Behav 9:372–378.

Varpe Ø, Jørgensen C, Tarling GA, Fiksen Ø (2009) The adaptive value of energy storage and capital breeding in seasonal environments. Oikos 118:363–370.

Vasemägi A, Primmer CR (2005) Challenges for identifying functionally important genetic variation: the promise of combining complementary research strategies. Mol Ecol 14:3623-3642.

Veasey J, Metcalfe N, Houston D (1998) A reassessment of the effect of body mass upon flight speed and predation risk in birds. Anim Behav 56:883–889.

Via S (1995) Adaptive phenotypic plasticity: consensus and controversy. Trends Ecol Evol 10:212–217.

Waddington C (1953) The "'Baldwin effect,'" "'genetic assimilation'" and "'homeostasis'." Evolution 7:386–387.

Wald G (1946) The metamorphosis of visual system in Amphibia. Biol Bull 91:239.

Walker JA (1997) Ecological morphology of lacustrine threespine stickleback *Gasterosteus aculeatus* L. (Gasterosteidae) body shape. Biol J Linn Soc 61:3–50.

Walker JA, Bell MA (2000) Net evolutionary trajectories of body shape evolution within a microgeographic radiation of threespine sticklebacks (*Gasterosteus aculeatus*). J Zool 252:293–302.

Walker JA, Ghalambor CK, Griset OL, McKenney D, Reznick DN (2005) Do faster starts increase the probability of evading predators? Funct Ecol 19:808–815.

Ward RD, Woodwark M, Skibinski DOF (1994) A comparison of genetic diversity levels in marine, freshwater, and anadromous fishes. J Fish Biol 44:213–232.

Ward AJW, Webster MM, Hart PJB (2006) Intraspecific food competition in fishes. Fish Fish 7:231–261.

Wark AR, Peichel CL (2010) Lateral line diversity among ecologically divergent threespine stickleback populations. J Exp Biol 213:108–117.

Webb JF (1989) Gross morphology and evolution of the mechanoreceptive lateral-line system in teleost fishes. Brain Behav Evol 33:34–53.

Weber JN, Peterson BK, Hoekstra HE (2013) Discrete genetic modules are responsible for complex burrow evolution in *Peromyscus* mice. Nature 493:402–405.

Werner EE, Gilliam JF (1984) The ontogenetic niche and species interactions in size structured populations. Ann Rev Ecol Syst 15:393–425.

Werner EE, Anholttt BR, Arbor A (1993) Ecological consequences of the trade-off between growth and mortality rates mediated by foraging activity. Am Nat 142:242–272.

West-Eberhard MJ (2003) Developmental Plasticity and Evolution. Oxford University Press, Oxford

West-Eberhard MJ (2005) Developmental plasticity and the origin of species differences. Proc Natl Acad Sci USA 102:6543–6549.

White SA, Fernald RD (1993) Gonadotropin releasing hormone-containing neurons change size with reproductive state in female *Haplochromis burtoni*. J Neurosci 13:434–441.

Whitman DW, Agrawal AA (2009) What is phenotypic plasticity and why is it important? In: Whitman DW, Ananthakrishnan TN (eds.) Phenotypic Plasticity of Insects. Science Publishers, Enfield, New Hampshire, pp 1–63

Wilson DS (1975) The adequacy of body size as a niche difference. Am Nat 109:769–784.

Wilson ADM, McLaughlin RL (2010) Foraging behaviour and brain morphology in recently emerged brook charr, (*Salvennius fontinalis*). Behav Ecol Sociobiol 64:1905-1914.

de Winter D, Oxnard CE (2001) Evolutionary radiation and convergences in the structural organization of mammalian brains. Nature 409:710-714.

Wirén A, Wright D, Jensen P (2013) Domestication-related variation in social preferences in chickens is affected by genotype on a growth QTL. Genes Brain Behav 12:330–337.

Wisenden BD, Chivers DP (2006) The role of public chemical information in anti-predator behaviour. In: Ladich F, Collins SP, Moller P, Kapoor B (eds.) Communication in Fishes, Science Publisher, Enfield, New Hampshire, pp 259–278

van Woerden JT, Willems EP, van Schaik CP, Isler K (2012) Large brains buffer energetic effects of seasonal habitats in catarrhine primates. Evolution 66:191–199.

Wolf M, Weissing FJ (2010) An explanatory framework for adaptive personality differences. Phyl Trans R Soc B 365:3959-3968.

Wootton RJ (1973) The effect of size of food ration on egg production in the female three-spined stickleback, *Gasterosteus aculeatus*, L. J Fish Biol 5:89–96.

Wootton RJ (1976) The Biology of the Sticklebacks. Academic Press, London

Wootton RJ (1984) A Functional Biology of Sticklebacks. University of California Press, Berkeley

Wright S. (1978) Variability within and among populations. In: Wright S (ed.) Evolution and the Genetics of Populations. University of Chicago Press, Chicago, IL, pp 336–347

Wright D, Butlin RK, Carlborg O (2006a) Epistatic regulation of behavioural and morphological traits in the zebrafish (*Danio rerio*). Behav Genet 36:914–922.

Wright D, Nakamichi R, Krause J, Butlin RK (2006b) QTL analysis of behavioral and morphological differentiation between wild and laboratory zebrafish (*Danio rerio*). Behav Genet 36:271–284.

Wund MA, Baker JA, Clancy B, Golub JL, Foster SA (2008) A test of the "flexible stem" model of evolution: ancestral plasticity, genetic accommodation, and morphological divergence in the threespine stickleback radiation. Am Nat 172:449–462.

Xu H, Shen X, Zhou M, Fang M, Zeng H, Nie Q, Zhang X (2010) The genetic effects of the dopamine D1 receptor gene on chicken egg production and broodiness traits. BMC Genet 11:17.

Yokoyama S, Yokoyama R (2000) Comparative molecular biology of visual pigments. In Stavenga DG, DeGrip WJ, Pugh EN (eds.) Handbook of Biological Physics, Vol. 3: Molecular Mechanisms in Visual Transduction. Elsevier, Amsterdam, pp 257-296

Yoshizawa M, Jeffery WR (2011) Evolutionary tuning of an adaptive behavior requires enhancement of the neuromast sensory system. Commun Integr Biol 4:89–91.

Yoshizawa M, Goricki S, Soares D, Jeffery WR, Gorički Š (2010) Evolution of a behavioral shift mediated by superficial neuromasts helps cavefish find food in darkness. Cur Biol 20:1631–1636.

Zelditch ML, Swiderski DL, Sheets HD, Fink WL (2004) Geometric Morphometrics for Biologists. Elsevier Academic Press, San Diego, CA

Ziuganov VV, Zotin AA (1995) Pelvic girdle polymorphism and reproductive barriers in the ninespine stickleback *Pungitius pungitius* (L.) from northwest Russia. Behaviour 132:1095-1105.

Zupanc GKH (2001) Adult neurogenesis and neuronal regeneration in the central nervous system of teleost fish. Brain Behav Evol 58:250-275.

Zupanc GKH (2006) Neurogenesis and neuronal regeneration in the adult fish brain. J Comp Physiol A 192:649-670.

dc_892_14

9. Függelék

A függelékben szereplő publikációk a 7. pontban megadott sorrendet követik.

dc_892_14

The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2012, **107**, 521–528. With 2 figures

Body size divergence in nine-spined sticklebacks: disentangling additive genetic and maternal effects

NURUL IZZA AB GHANI*, GÁBOR HERCZEG and JUHA MERILÄ

Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, PO Box 65, FI-00014 Helsinki, Finland

Received 3 April 2012; revised 2 May 2012; accepted for publication 2 May 2012

Interpopulation differences in body size are of common occurrence in vertebrates, but the relative importance of genetic, maternal, and environmental effects as causes of observed differentiation have seldom been assessed in the wild. Gigantism in pond nine-spined sticklebacks (*Pungitius pungitius* Linnaeus, 1758) has been repeatedly observed, but the quantitative genetic basis of population divergence in size has remained unstudied. We conducted a common garden experiment – using 'pure' and reciprocal crosses between two populations ('giant' pond versus 'normal' marine) – to test for the relative importance of additive genetic, non-additive genetic, and maternal effects on body size after 11 months of growth in the laboratory. We found that body size difference between the two populations in laboratory conditions owed mainly to additive genetic effects, and only to a minor degree to maternal effects. Furthermore, the weak maternal effects were seen only in the offspring of 'giant' mothers, and appeared to be mediated through differences in egg size. Thus, the results suggest that gigantism in pond populations of *P. pungitius* is based on the effects of additively acting genes, rather than to direct environmental induction, or maternal or non-additive gene action. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **107**, 521–528.

ADDITIONAL KEYWORDS: genetics – geographic variation – gigantism – maternal effect.

INTRODUCTION

Evolution of body size is a central theme in many studies of adaptation and population differentiation because body size often influences – or correlates with – other aspects of morphology, performance, fitness, and physiology of individuals (Shine, 1988, 1989; Blanckenhorn, 2000; Roff, 2002). Intraspecific variation in body size is commonly observed among geographically separated populations (e.g. James, 1970; Endler, 1977; Ashton, Tracy & de Queiroz, 2000), and the genetic basis to this divergence has also been demonstrated in a number of cases (e.g. Stearns, 1983; Gilchrist & Partridge, 1999; Lynch *et al.*, 1999). However, the relative importance of contributions from additive genetic, non-additive genetic, and maternal and environmental effects on extreme size divergence within vertebrate species – such as the gigantism observed in nine-spined sticklebacks (*Pungitius pungitius* Linnaeus, 1758; Herczeg, Gonda & Merilä, 2009) – have seldom been explored. One reason for this is that reciprocal crosses between geographically separated populations are needed to evaluate the relative contributions of additive genetic, non-additive genetic, and maternal and environmental effects (Lynch & Walsh, 1998), and such studies are logistically demanding to conduct. Yet, they have been conducted in plants (e.g. Mazer, 1987), crustaceans (e.g. Allan, 1984), insects (e.g. Azevedo, French & Partridge, 1997), and also in a few vertebrate species (e.g. Berven, 1982; Harris, Fondacaro & Kasbohm, 1995; Laugen, Laurila & Merilä, 2002).

The genetic contribution to geographic differentiation consists of both independent effects of parental genotypes (additive genetic effects) and their interaction (i.e. non-additive genetic effects, such as dominance and epistasis), but maternal effects may also be partly genetically determined (Lynch & Walsh, 1998). Maternal effects have been demonstrated to have significant ecological and evolutionary consequences (Kaplan, 1998; Mousseau & Fox, 1998; Green, 2008). They can be mediated by the environment (e.g. Mousseau & Fox, 1998; Rossiter, 1998; Einum & Fleming, 2002), or can act through material resources such as nutrients, mRNAs, and immunoproteins that are supplied by the mother through lactation (e.g. Oksh, Sutherland & Williams, 1967; Cowley *et al.*, 1989; Green, 2008), in eggs (e.g. Laugen *et al.*, 2002; Green, 2008), or through the placenta (Cowley *et al.*, 1989). Whether mediated through the environment or material resources, the environmental conditions experienced by mothers may affect the offspring's performance over several generations (Rossiter, 1996; Lacey, 1998; Green, 2008). For this reason, it is recommended (e.g. Lynch & Walsh, 1998: 123–127) that offspring should be raised under common environmental conditions for a few generations, but this approach may not be possible in animals with long generation times. Thus, another option (see also Roth & Klein, 1986) is to do reciprocal crosses between geographically separated populations and compare trait values in the different hybrid and pure crosses (e.g. Azevedo *et al.*, 1997; Laugen *et al.*, 2002).

The main aim of this study was to explore the genetic basis of intraspecific body size divergence in *P. pungitius* using a common garden breeding design, allowing the disentanglement of additive genetic effects from maternal, and to some degree also from non-additive genetic effects. This species is a particularly well-suited model for this purpose, as gigantism has been observed in several independent pond populations lacking piscine predators, whereas lake and marine populations sympatric with piscine predators are not known to reach gigantic body size (Herczeg *et al.*, 2009; Herczeg, Gonda & Merilä, 2010; Herczeg *et al.*, 2012; Mobley *et al.*, 2011). We crossed males and females from one marine population (i.e. 'normal'-sized fish) and one pond population (i.e. with fish reaching 'giant' body size) in a design that included both 'pure' (pond–pond and marine–marine) crosses and their reciprocal 'hybrids' (pond–marine and marine–pond). If the body size differences among populations are mainly caused by additive genetic effects, we expect that F_1 'pure' crosses will differ in their mean body size, and both types of 'hybrid' crosses will be intermediate in size to 'pure' crosses (Wright, 1978). If maternal and/or non-additive genetic effects were important, we would expect to see 'hybrid' crosses to deviate from expected intermediacy. For instance, under simple dominance, we would expect the 'hybrids' to be closer to one of the 'pure' lines (the one possessing the dominant alleles), whereas maternal effects might result in 'hybrid' crosses being closer to their mothers' 'pure' line. In addition to a focus on genetic and maternal effects in body size, we also investigated population differences in mean egg size, and potential effects of egg size on the observed patterns.

MATERIAL AND METHODS

Sampling, breeding, and rearing

Adult *P. pungitius*, which formed the parental generation, were collected from the wild during the early phase of the reproductive season in late May–mid June of 2010. Two populations were sampled: a marine population (Baltic Sea at Helsinki, 60°12′09″ N, 25°10′58″ E) and a pond population (Pyöreälampi; 66°15′40″ N, 29°26′00″ E), separated by ~900 km. Sampling was performed using minnow traps (Pyöreälampi) and a seine net (Helsinki). The marine site was a shallow coastal, brackish water bay with low salinity. Pyöreälampi is an isolated pond (surface area < 5 ha) in which *P. pungitius* is the only fish species (apart from the recently introduced whitefish *Coregonus lavaretus* Linnaeus, 1758 that is present in very low density). The two populations are isolated both geographically and genetically (neutral molecular marker divergence $F_{ST} = 0.46$; Shikano *et al.*, 2010; Shimada *et al.*, 2011). The Pyöreälampi population consists of 'giant' sticklebacks (with the total length of a fish aged 5+ years being ~8–10 cm, and with the largest fish we caught being > 11 cm in length), whereas the Helsinki fish are 'normal' sized (usually < 5 cm in length; Herczeg *et al.*, 2009, 2010).

Some of the artificial crosses were performed at the site of capture, whereas some of them were performed in the laboratory. This means that conditions for the fertilized eggs were not fully identical for the first 2 days of egg development (time between fertilization and hatching was *c.* 6 days). However, as we measured fish size almost a year after hatching (see below), we assume that any effect stemming from this difference is negligible. Four different types of crosses were produced: two 'pure' crosses by crossing either Helsinki males with Helsinki females (hereafter HH) or Pyöreälampi males with Pyöreälampi females (hereafter PP), and two 'hybrid' crosses by crossing either Pyöreälampi males with Helsinki females (hereafter PH) or Helsinki males with Pyöreälampi females (hereafter HP). Crosses were made *in vitro* between randomly chosen males and females by gently squeezing the eggs out from the ripe females and pouring the sperm solution on the eggs. The sperm solution was obtained by mincing the testicles of over-anaesthetized males in a drop of water. A total of 40 full-sib families (ten per cross-type) were produced, and each parent was used for just one cross.

Fish (five individuals per family) were reared individually in four 1.4-l tanks (Allentown Zebrafish Rack

*Corresponding author. E-mail: abghani@mappi.helsinki.fi

Systems, hereafter refereed to as racks; Aquaneering Inc., San Diego, CA, USA) equipped with physical, biological, chemical, and UV filters. Freshwater (salinity 0 psu) was used for rearing, and therefore no osmoregulation-related issues should contribute to differences in growth rate, as the parental fish from the Baltic Sea coast originate from water of very low salinity (0–6.0 psu; Shimada *et al.*, 2011). Fish were fed *ad libitum* with live brine shrimp (*Artemia* sp.) nauplii for the first two months of their life, and with frozen bloodworms thereafter. They were kept under a 14-h light/10-h dark photoperiod, with the water temperature held at 17 °C throughout the experiment. Three hundred days after hatching, all fish were subjected to artificial hibernation (in order to facilitate reproduction for other scientific purposes), in which the temperature and light regime was gradually shifted towards a 24-h dark photoperiod and water temperature of 4 °C within 2 weeks. The artificial overwintering lasted for 30 days and fish size was measured after that. The length of the available growth period ensured that fish from all crosses approached their adult size (Herczeg *et al.*, 2012). The survival rate during the experiment was high: more than 90% of individuals in each type of cross (HH, 90%; HP, 98%; PH, 92%; PP, 92%) survived to the end of the experiment.

Measurements

Twenty eggs per family from all crosses were measured to get an estimate of egg size for mothers. The measurements were made from photographs taken of eggs after fertilization with size reference using a Nikon D60 digital camera. The egg size was measured as the diameter of the eggs (to the closest 0.01 mm) using the program TPSDIG 2 (Rohlf, 2002). HH eggs were photographed just after fertilization, whereas the eggs from other crosses were photographed 2 days after fertilization because of logistic constraints associated with the transportation of the eggs. To verify that egg size does not change within the 2-day interval, we conducted a small pilot study. Two families per cross types (HH, PH, HP, and PP) were made, and we measured 20 eggs per family from all crosses immediately after fertilization and again 2 days later. We analysed the possible egg size change within this 2-day interval after fertilization with general linear mixed models (GLMMs) treating egg size as a dependent variable, cross and time as fixed factors (note that we did not follow eggs individually), and family nested within cross as a random factor. We found that egg size differed among crosses, but neither time nor cross × time affected egg size (cross, $F_{3,4} = 177.35$, $P < 0.001$; time, $F_{1,148} = 1.56$, $P = 0.21$; cross × time, $F_{3,148} = 1.49$, $P = 0.22$). Family within each cross did not affect egg size either ($Z = 1.15$, $P = 0.25$). Hence, we believe that the 2 days shift in egg size measurements did not cause any bias, and that the use of the original egg size measurements was justified.

The data set consisted of 188 individuals (HH, 45; HP, 48; PH, 49; PP, 46). All individuals were photographed (alive) at 337 days after hatching, i.e. 1 week after the end of the artificial hibernation (when the temperature and light regime started to change). All photographs were taken with digital camera (Nikon D60), with a ruler used as a size reference in the photographs. Fish size was measured as standard length from the tip of the lower jaw to the base of the caudal peduncle (to the closest 0.01 mm), also using the TPSDIG 2 (Rohlf, 2002).

Statistical analyses

To see if there were differences in body size, mean egg size, and clutch size between Helsinki and Pyöreälampi females, we analysed these variables with general linear models (GLMs), treating them as dependent variables, and treating their population of origin as a fixed effect. To see whether there were size-independent mean egg size and clutch size trends between the populations, we also ran GLMs with female body size added as a covariate.

To investigate the relative influence of additive genetic, maternal, and non-additive genetic effects on body size, a 2 × 2 factorial design was used in which the factors (fixed) were the male and female populations of origin. These data were analysed with GLMMs treating body size as the dependent variable, male and female origin (Helsinki versus Pyöreälampi) and their interaction as fixed factors, and family nested within male origin × female origin as a random factor. Post-hoc pairwise comparisons were performed with Fisher's tests. We ran two models, either with or without mean egg size in the given family added as a covariate. The logic of including egg size was that many – but not all – of the maternal effects might correlate with egg size (Kaplan, 1998; Laugen *et al.*, 2002; Green, 2008). Hence, if maternal effects were still detectable after accounting for egg size, this would be evidence for egg size-independent maternal effects. However, when egg size as a covariate and its interactions with the fixed factors were added to the model, model selection became a necessity (e.g. Engqvist, 2005). Hence, we applied a backward stepwise model selection based on the $P < 0.05$ criterion, which is considered a conservative method (Murtaugh, 2009). Briefly, we started with the full factorial model and then removed the non-significant terms, starting with the highest level interactions and ending with the main effects. The main effects included in significant interactions were not removed.

We also performed variance component analyses (VCAs) to assess quantitatively the relative importance of the factors used in the GLMMs. In the first VCA, body size was the dependent variable, and male origin, female origin, their interaction, and family nested within male origin × female origin were entered as random factors. In the second VCA, we added egg size as a covariate (fixed effect) without interactions. Variance components were estimated with the restricted maximum likelihood (REML) method.

To illustrate the degree of phenotypic differentiation of F_1 'hybrids' from the 'pure' crosses, we also performed an analysis of intermediacy ($\Delta F_1/\Delta U$ indices) following Wright (1978; see also Laugen *et al.*, [2002]). ΔF_1 refers to the mean of the given F_1 and ΔU refers to the difference between the means of the two 'pure' crosses. A value of 0.50 indicates an exact intermediate F_1 'hybrid' phenotype. Deviations from this value are indicative of non-additive genetic or maternal effects. We compared the size of 'hybrids' with the expected value under perfect intermediacy (indicative of only additive genetic effects) with one-sample Student's *t*-tests. All analyses were carried out in PASW Statistics 18 (PASW Inc., 2009).

RESULTS

Female size and reproductive output

Pyöreälampi and Helsinki populations differed in female size ($F_{1,38} = 388.45$, $P < 0.001$; Fig. 1A), egg size ($F_{1,38} = 261.73$, $P < 0.001$; Fig. 1B), and clutch size ($F_{1,38} = 16.29$, $P < 0.001$; Fig. 1C). However, when controlling for female size, the differences in mean egg and clutch size disappeared (egg size, $F_{1,36} = 0.02$, $P = 0.89$; clutch size, $F_{1,36} = 0.23$, $P = 0.64$), suggesting that the differences in reproductive output were directly related to the population divergence in female size.

Size after hibernation

Mean body size at the end of the experiment differed markedly among the four different cross types: HH offspring were the smallest, PP offspring were the largest, and offspring from the two 'hybrid' crosses were intermediate in body size (Fig. 2). The effects of male and female origin were both highly significant (male origin, $F_{1,36} = 52.61$, $P < 0.001$; female origin, $F_{1,36} = 113.54$, $P < 0.001$), suggesting an additive genetic basis for population differences in size. The marginally significant male origin × female origin interaction ($F_{1,36} = 3.88$, $P < 0.056$) suggested weak non-additive genetic and/or maternal effects. Post-hoc tests revealed that all pairs of the four crosses differed significantly from each other ($P < 0.02$ in all tests). The family effect was non-significant ($Z = 1.34$, $P = 0.18$). The significant difference between the two 'hybrid' crosses provided more support for the presence of maternal or/and non-additive genetic effects.

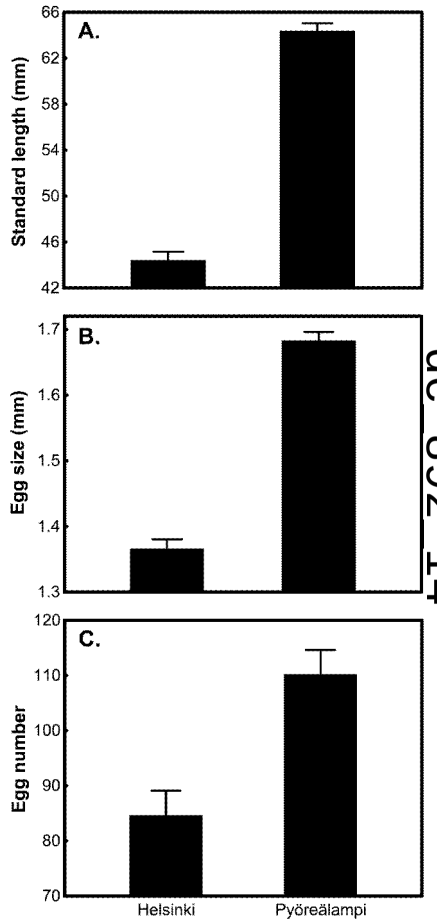


Figure 1. Population divergence in (A) female size, (B) egg size, and (C) number of eggs between marine (Helsinki, Baltic Sea) and pond (Pyöreälampi) female nine-spined sticklebacks (*Pungitius pungitius*). Data presented are means ± SEs.

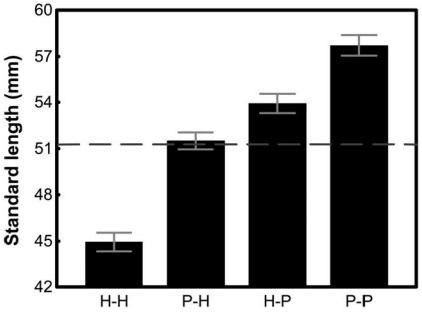


Figure 2. Differences in mean size (±SE) 337 days after hatching among the pure and hybrid nine-spined stickleback (*Pungitius pungitius*) crosses. 'H' denotes a Helsinki parent and 'P' denotes a Pyöreälampi (pond) parent; the first letter denotes the father and the second the mother. The horizontal dashed line represents the expected value for hybrids under perfect intermediacy.

Analyses of intermediacy suggested that although the PH hybrids (0.51) hardly differed from what was expected under perfect intermediacy (i.e. 0.5), HP hybrids did so (0.70), and occupied a position almost equally distant from the expected intermediacy and the pure PP cross (Fig. 2). This asymmetric divergence from intermediacy was also confirmed statistically: HP hybrids differed significantly from the expected mean size between HH and PP crosses ($t_{47} = 4.12$, $P < 0.001$), whereas PH hybrids did not ($t_{48} = 0.37$, $P = 0.71$; Fig. 2). This contradicts the expectations under simple dominance, and is suggestive of relatively weak (compared with the additive genetic effect) maternal effects transmitted by Pyöreälampi, but not by Helsinki females. VCA revealed that the factors in the GLMM explained 73% of the total variance in size: of this, family effect accounted for 3.8%, male origin 28.6%, female origin 64.3%, and the interaction between male and female origin 3.4% of the total variance. Hence, the population origin of parents accounted for more variation than their identity (i.e. family effect), and the origin of dams was more than two times as important as the origin of sires, giving further support for the existence of maternal effect influences.

When egg size was included as a covariate into the GLMM model, the male origin × female origin interaction became non-significant, suggesting that maternal effects might be partly explained as maternal investments in egg size, even though the egg size effect itself was non-significant (Table 1). Here, post-hoc tests (based on a model with male origin, female origin, their interaction, and egg size) showed that the hybrids did not differ significantly in size ($P = 0.55$), whereas the other pairs did (all $P < 0.003$). VCA corrected for egg size showed that factors in the second GLMM explained 66% of variance, of which the family term accounted for 5.5%, male origin accounted for 40.8%, female origin accounted for 51%, and the interaction between male and female origin accounted for 2.6% of the total variance. The main difference from the previous VCA results was the drastic decrease in the difference between variance explained by males versus females, suggesting that the maternal effect was at least partly expressed via egg size.

Table 1. Results of the general linear mixed model of nine-spined stickleback (*Pungitius pungitius*) body size on origin of female and male and mean egg size in a clutch. A backward stepwise model selection was applied, and non-significant terms are shown as seen at removal. The family effect was non-significant ($Z = 1.34$, $P = 0.18$).

Source	df_1, df_2	F	P
Male origin	1, 37.11	48.93	< 0.0001
Female origin	1, 37.10	105.61	< 0.0001
Egg size	1, 35.26	1.25	0.27
Male origin × female origin	1, 33.40	0.002	0.96
Male origin × egg size	1, 35.22	2.95	0.09
Female origin × egg size	1, 34.09	0.19	0.66
Male origin × female origin × egg size	1, 33.45	0.17	0.68

DISCUSSION

Whereas intraspecific size variation and even gigantism has been described from many different species (e.g. Forsman, 1991; Ford, 2005; Keogh, Scott & Hayes, 2005; Lomolino, 2005; Herczeg *et al.*, 2009), the genetic and environmental bases of this phenomenon have been rarely studied in detail. Extreme size differences can result from simple environmental effects such as population differences in resource availability and/or, especially in the case of taxa with indeterminate growth, differences in longevity (Roff, 2002). Even when size differences remain in the F_1 generation reared under common garden conditions, the contribution of maternal and environmental effects cannot be automatically ruled out. This is because maternal or cross-generational effects can influence offspring size even in the F_2 generation (e.g. Rossiter, 1996; Lynch & Walsh, 1998). However, as the results show, there was only evidence for weak maternal effects in the F_1 generation, and hence, it is also unlikely that these effects would be found in any subsequent generations.

In *P. pungitius*, gigantism in the wild appears to result from the combined effects of extended longevity and an increased year-to-year growth rate in pond populations living in a predator-free environment (Herczeg *et al.*, 2009). The larger body size of pond, as compared with marine, *P. pungitius* from the very same study populations as were used in this study have been confirmed previously in laboratory studies (Herczeg *et al.*, 2009, 2012; Shimada *et al.*, 2011). However, none of these studies have used inter-population crosses to explore how much of this divergence could arise from cross-generational environmental or non-additive genetic effects. Here, we took a step forward in this respect, and verified that the extreme size divergence (quantitative trait divergence QST = 0.973 ± 0.063 SE; Shimada *et al.*, 2011) between the pond and marine *P. pungitius* populations has a strong genetic component, likely to owe largely to additive genetic effects. Whereas the breeding design applied in this study allowed an evaluation of the relative importance of additive genetic versus maternal (and non-additive genetic) contributions to body size divergence, it is still limited in estimating more complex gene interactions and the possible contributions of inherited maternal effects. Thus, to study these effects, one needs line cross-analyses based on multiple segregation generations (Lynch & Walsh, 1998). However, as only one of the 'hybrid' crosses in this study displayed a weak deviation from the expectation under a pure additive model of inheritance, the importance of such high-order interactions in the data are likely to be small, but perhaps not negligible (see below).

Although the results unravelled little evidence for maternal and non-additive genetic effects as being important in determining body size differences among the two populations, it is interesting that the analyses of intermediacy revealed a tendency for one of the 'hybrid' crosses to deviate from perfect intermediacy. Whereas 'hybrids' from mothers of Helsinki origin were perfectly intermediate to the 'pure' crosses, the 'hybrids' from mothers of Pyöreälampi origin were close to halfway between the expected intermediate phenotype and the mean of the 'pure' ('giant') Pyöreälampi crosses. Hence, Pyöreälampi females seemed to influence the size of their offspring in ways that cannot be easily explained by additive genetic effects, and it may suggest either non-additive gene action (but not simple dominance) or interaction between genetic and maternal effects (cf. Rossiter, 1998). The fact that adding egg size into the analyses diminished the interaction effect suggests that this deviation might be due to egg size-related maternal effects. It is also possible that the genes involved in determining body size in the Pyöreälampi population were expressed differently depending on which maternal environmental background they resided. However, given the lack of firm statistical evidence for maternal or non-additive genetic effects (i.e. a marginally significant interaction effect), the most parsimonious interpretation of this finding is that the observed body size differences between the two populations are mainly the result of additive genetic differences among these populations. Hence this experiment lends support for the earlier interpretations that the divergence in body size among these populations is genetically based (Herczeg *et al.*, 2009, 2010, 2012; Shimada *et al.*, 2011).

In accordance with an earlier study (Herczeg *et al.*, 2010), we found that the mean female body size and reproductive output of wild-caught females was radically different between Helsinki and Pyöreälampi populations: 'giant' females from Pyöreälampi produced more and larger eggs than 'normal'-sized females from Helsinki. The egg size difference between populations can be explained by differences in female body size: once the female size was controlled for, egg size differences between the populations disappeared. Maternal effects mediated through egg size have been identified as a significant source of variation in hatchlings (e.g. Heath, Fox & Heath, 1999; Hendry & Day, 2003), and even in adult traits (reviewed in Mousseau & Fox, 1998; Green, 2008), in many organisms. However, maternal effects on offspring body size after almost 1 year from hatching were barely detectable in our study. Furthermore, even this weak effect vanished when the egg size differences, which were not affecting offspring size per se, were controlled for. Therefore, egg size itself is unlikely to explain the variation in *P. pungitius* body size after their first hibernation.

Finally, we note that the fish in the experiments reported in this study did not express as large a body size divergence as the wild fish from the same localities (cf. Herczeg *et al.*, 2009). This is not surprising given the fact the laboratory-reared fish were less than 1 year of age when measured. At this point the Helsinki fish have already reached a body size close to their asymptotic size, whereas the Pyöreälampi fish are still in the fast growth phase of their growth trajectory (Shimada *et al.*, 2011; Herczeg *et al.*, 2012). Hence, the fact that the body size differences in our common garden experiment are smaller than those observed among wild-caught fish does not constitute evidence to show that the body size differences in the wild would be driven by environmental effects. Giant *P. pungitius* in the wild are often more than 5 years old, and although environmental effects are likely to

contribute to their growth (Herczeg *et al*., 2009), a genetic contribution to this gigantism is indicated by our present and earlier results showing divergent growth trajectories for the fish from pond and marine habitats (Shimada *et al*., 2011; Herczeg *et al*., 2012). Yet, it is possible that the genetically determined high growth rate (and extended growth period; Herczeg *et al*., 2012) in the pond fish are also influenced by environmental effects (stemming from a negligible predation risk), resulting in gigantism. If so, the realized gigantism can be a combined result of genetic divergence (G), direct environmental effects (E), and genotype × environment interactions (G × E). Our present study revolves around G, and thus we have no data of the relative contributions of G, E, and G × E to the extreme size divergence between *P. pungitius* populations in the wild. This is a common shortcoming of common garden studies where testing is performed in one environment only, but we have no a priori reason to think that our results would be different had the rearing been performed under different environmental conditions.

In conclusion, the results suggest that the extreme size divergence seen in these two geographically distinct *P. pungitius* populations has an additive genetic component, and if not negligible, the contributions of maternal and non-additive genetics to this differentiation are small. This is an important finding in the view that the assumption about the genetic basis of gigantism in pond populations of this species (cf. Herczeg *et al*., 2009) is now confirmed. Hence, the *P. pungitius* system is a promising candidate model in identifying the genes responsible for the evolution of gigantism, especially in view of the genomic resources that have recently been developed for this species (e.g. Shapiro *et al*., 2009).

ACKNOWLEDGEMENTS

We thank Marika Lilja, Linda Uoti, and Mirva Turtiainen for their help during the experiments, EGRU members and four anonymous reviewers for their helpful comments on earlier versions of this paper. The research was supported by the Academy of Finland (grants #129662, #134728 and #218343 to JM and #128716 to GH), as well as from the Universiti Putra Malaysia (to IAG). The research leading to these results has also received funding from the European Community's Seventh Framework Programme (FP/2007–2013) under grant agreement n°217246 through BONUS, the joint Baltic Sea research and development programme (to JM). The experiments were conducted with a license from the Finnish National Animal Experiment Board (license no. STH211A).

REFERENCES

Allan JD. 1984. Life history variation in a freshwater copepod: evidence from population crosses. *Evolution* **38:** 280–291.

Ashton KG, Tracy MC, de Queiroz A. 2000. Is Bergmann's rule valid for mammals? *American Naturalist* **156:** 390–415.

Azevedo RBR, French V, Partridge L. 1997. Life-history consequences of egg size in *Drosophila melanogaster*. *American Naturalist* **150:** 250–282.

Berven KA. 1982. The genetic basis of altitudinal variation in the wood frog *Rana sylvatica* II. An experimental analysis of larval development. *Oecologia* **52:** 360–369.

Blanckenhorn WU. 2000. The evolution of body size: what keeps organisms small? *The Quarterly Review of Biology* **75:** 385–407.

Cowley DE, Pomp D, Atchley WR, Eisen EJ, Hawkins-Brown D. 1989. The impact of maternal uterine genotype on postnatal growth and adult body size in mice. *Genetics* **122:** 193–203.

Einum S, Fleming IA. 2002. Does within-population variation in fish egg size reflect maternal influences on optimal values? *American Naturalist* **160:** 754–765.

Endler JA. 1977. *Geographic variation, speciation and clines*. Princeton, NJ: Princeton University Press.

Engqvist L. 2005. The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Animal Behaviour* **70:** 967–971.

Ford SM. 2005. Subfossil platyrrhine tibia (Primates: Callitrichidae) from Hispaniola: a possible further example of Island gigantism. *American Journal of Physical Anthropology* **70:** 47–62.

Forsman A. 1991. Variation in sexual size dimorphism and maximum body size among adder populations: effects of prey size. *Journal of Animal Ecology* **60:** 253–267.

Gilchrist AS, Partridge L. 1999. A comparison of the genetic basis of wing size divergence in three parallel body size clines of *Drosophila melanogaster*. *Genetics* **153:** 1775–1787.

Green BS. 2008. Maternal effects in fish populations. *Advances in Marine Biology* **54:** 1–105.

Harris RN, Fondacaro JV, Kasbohm LA. 1995. Local variation in the genetic basis of morphological traits in the salamander *Ambystoma talpoideum*. *Evolution* **49:** 565–569.

Heath DD, Fox CW, Heath JW. 1999. Maternal effects on offspring size: variation through early development of Chinook salmon. *Evolution* **53:** 1605–1611.

Hendry AP, Day T. 2003. Revisiting the positive correlation between female size and egg size. *Evolutionary Ecology Research* **5:** 421–429.

Herczeg G, Gonda A, Kuparinen A, Merilä J. 2012. Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? *Evolutionary Ecology* **26:** 109–122.

Herczeg G, Gonda A, Merilä J. 2009. Evolution of gigantism in nine-spined sticklebacks. *Evolution* **63:** 3190–3200.

Herczeg G, Gonda A, Merilä J. 2010. Rensch's rule inverted – female-driven gigantism in nine-spined stickleback *Pungitius pungitius*. *Journal of Animal Ecology* **79:** 581–588.

James FC. 1970. Geographic size variation in birds and its relationship to climate. *Ecology* **51:** 365–390.

Kaplan RH. 1998. Maternal effects, developmental plasticity and life history evolution. In: Mousseau TA, Fox CW, eds. *Maternal effects as adaptations*. New York, NY: Oxford University Press, 244–260.

Keogh JS, Scott IAW, Hayes C. 2005. Rapid and repeated origin of insular gigantism and dwarfism in Australian tiger snake. *Evolution* **59:** 226–233.

Lacey EP. 1998. What is an environmentally induced parental effect? In: Mousseau TA, Fox CW, eds. *Maternal effects as adaptations*. New York, NY: Oxford University Press, 54–66.

Laugen AT, Laurila A, Merilä J. 2002. Maternal and genetic contributions to geographical variation in *Rana temporaria* larval life-history traits. *Biological Journal of the Linnean Society* **76:** 61–70.

Lomolino MV. 2005. Body size evolution in insular vertebrates: generality of the island rule. *Journal of Biogeography* **32:** 1683–1699.

Lynch M, Pfrender M, Spitze K, Lehman N, Hicks J, Allen D, Latta L, Ottene M, Bogue F, Colbourne J. 1999. The quantitative and molecular genetic architecture of a subdivided species. *Evolution* **53:** 100–110.

Lynch M, Walsh B. 1998. *Genetics and analysis of quantitative traits*. Massachusetts: Sunderland.

Mazer SJ. 1987. The quantitative genetics of life history and fitness components in *Raphanus raphanistrum* L. (Brassicaceae): ecological and evolutionary consequences of seed weight variation. *American Naturalist* **130:** 891–914.

Mobley KB, Lussetti D, Johansson F, Englund G, Bokma F. 2011. Morphological and genetic divergence in Swedish postglacial stickleback (*Pungitius pungitius*) populations. *BMC Evolutionary Biology* **11:** 287–305.

Mousseau TA, Fox CW. 1998. *Maternal effects as adaptations*. New York, NY: Oxford University Press.

Murtaugh PA. 2009. Performance of several variable-selection methods applied to real ecological data. *Ecology Letter* **12:** 1061–1068.

Oksh HA, Sutherland TM, Williams JS. 1967. Prenatal and postnatal maternal influence on growth in mice. *Genetics* **57:** 79–94.

PASW Inc. 2009. Chicago, Illinois. IBM PASW Statistics 18 Core System User's Guide.

Roff DA. 2002. *Life history evolution*. Massachusetts: Sinauer Associates, Inc. Sunderland.

Rohlf FJ. 2002. tpsDig, digitize landmarks and outlines, version 1.37. Department of Ecology and Evolution, State University of New York at Stony Brook.

Rossiter M. 1996. Incidence and consequences of inherited environmental effects. *Annual Review of Ecology and Systematics* **27:** 451–476.

Rossiter M. 1998. The role of environmental variation in parental effects expression. In: Mousseau TA, Fox CW, eds. *Maternal effects as adaptations*. New York, NY: Oxford University Press, 112–134.

Roth VL, Klein MS. 1986. Maternal effects on body size of large insular *Peromyscus maniculatus*: evidence from embryo transfer experiments. *Journal of Mammalogy* **67:** 37–45.

Shapiro MD, Summers BR, Balabhadra S, Aldenhoven JT, Miller AL, Cunningham CB, Bell MA, Kingsley DM. 2009. The genetic architecture of skeletal convergence and sex determination in ninespine sticklebacks. *Current Biology* **19:** 1140–1145.

Shikano T, Shimada Y, Herczeg G, Merilä J. 2010. History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. *Molecular Ecology* **19:** 1147–1161.

Shimada Y, Shikano T, Kuparinen A, Gonda A, Leinonen T, Merilä J. 2011. Quantitative genetics of body size and timing of maturation in two nine-spined stickleback (*Pungitius pungitius*) populations. *PLoS ONE* **6:** e28859.

Shine R. 1988. The evolution of large body size in females: a critique of Darwin's fecundity advantage model. *American Naturalist* **131:** 124–131.

Shine R. 1989. Ecological causes for the evolution of sexual size dimorphism: a review of the evidence. *Quarterly Review of Biology* **64:** 419–461.

Stearns SC. 1983. The genetic basis of differences in life-history traits among six populations of mosquitofish (*Gambusia affinis*) that shared ancestors in 1905. *Evolution* **37:** 618–627.

Wright S. 1978. Variability within and among populations. In: Wright S, ed. *Evolution and the genetics of populations*. Chicago, IL: Univ Chicago Press, 336–347.

JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12085

Evidence for genetic differentiation in timing of maturation among nine-spined stickleback populations

N. I. A. GHANI*, G. HERCZEG†, T. LEINONEN* & J. MERILÄ*

*Department of Biosciences, University of Helsinki, Helsinki, Finland
†Department of Systematic Zoology and Ecology, Eötvös Loránd University, Budapest, Hungary

Keywords:

dominance;
genetics;
geographic variation;
maturation;
Pungitius pungitius.

Abstract

Timing of maturation is an important life-history trait that is likely to be subjected to strong natural selection. Although population differences in timing of maturation have been frequently reported in studies of wild animal populations, little is known about the genetic basis of this differentiation. Here, we investigated population and sex differences in timing of maturation within and between two nine-spined stickleback (*Pungitius pungitius*) populations in a laboratory breeding experiment. We found that fish from the high-predation marine population matured earlier than fish from the low-predation pond population and males matured earlier than females. Timing of maturation in both reciprocal hybrid crosses between the two populations was similar to that in the marine population, suggesting that early timing of maturation is a dominant trait, whereas delayed timing of maturation in the pond is a recessive trait. Thus, the observed population divergence is suggestive of strong natural selection against early maturation in the piscine-predator-free pond population.

Introduction

Timing of maturation has important fitness consequences for animals and plants, and thus, it is expected to be under strong selection and optimized in different populations (e.g. Gibbons *et al.*, 1981; Stearns, 1984; Campton & Gall, 1988; Reznick *et al.*, 1990; Houle & Rowe, 2003; Reznick & Ghalambor, 2005). Selection should generally favour reproduction at the earliest physiologically possible age, especially in environments with high mortality rates (Stearns, 1992; Hernaman & Munday, 2005). However, trade-offs between reproduction and survival or current and future reproductive output might favour delayed maturation under certain circumstances (Reznick *et al.*, 1990; Roff, 1992, 2002; Stearns, 1992; Reznick & Ghalambor, 2005). Thus, variation in timing of maturation has been demonstrated both between and within animal and plant populations (Roff, 1992, 2002; Stearns, 1992, 2000; Heibo, 2003), but the evidence for the genetic basis of this variation – both among (Kallman *et al.*, 1973; Kallman & Borkoski, 1978; Berven, 1982; Quinn *et al.*, 2000; Quinton *et al.*, 2004) and within populations (Kolluru & Reznick, 1996; Charpentier *et al.*, 2008) – in vertebrates is still scarce.

This scarcity of evidence is understandable given the difficulty of disentangling the relative roles of genetic and environmental effects in timing of maturation due to confounding effects of environment (e.g. temperature, predation, photoperiod: Baggerman, 1985; Taranger *et al.*, 1999) and growth history (e.g. Gadgil & Bossert, 1970; Rijnsdorp, 1993; Morita & Fukuwaka, 2006). Yet, a better understanding of the genetic basis of variation in timing of maturation is important for many reasons. For instance, much of the debate around fisheries-induced evolution stems from the difficulty of separating the effects of genes and environment on the observed changes in timing of maturation (e.g. Law, 2000; Mollet *et al.*, 2007; Wright, 2007). Therefore, there is an urgent need to understand how shifts in timing of maturation are achieved in the wild. The nine-spined stickleback (*Pungitius pungitius*) is an excellent model to study this question. It is a small teleost fish suited well for laboratory studies, and both data from the wild and laboratory suggest that marine

Correspondence: Nurul Izza Ab Ghani, Ecological Genetics Research Unit, Department of Biosciences, PO Box 65, FI-00014 University of Helsinki, Helsinki, Finland. Tel.: +358 9 191 57710; fax: +358 9 191 57694; e-mail: abghani@mappi.helsinki.fi

nine-spined sticklebacks – subject to intense piscine predation – mature earlier than fish from isolated ponds lacking piscine predators (Herczeg *et al.*, 2009; Shimada *et al.*, 2011). Although these observations suggest adaptive genetic differentiation in timing of maturation among nine-spined stickleback populations, further work is required to verify the genetic basis of this differentiation.

The aim of this study was to investigate the patterns and genetic basis of timing of maturation in two phenotypically (Herczeg *et al.*, 2009, 2012; Shimada *et al.*, 2011) and genetically (Shikano *et al.*, 2010a; Shimada *et al.*, 2011; Bruneaux *et al.*, 2012) divergent nine-spined stickleback populations, making use of within and between population crosses grown under controlled laboratory conditions. We crossed males and females from one marine and one pond population in a design that included both 'pure' (marine marine and pond pond) crosses and their reciprocal 'hybrids' (marine male–pond female and pond male marine female). In particular, we were interested to see whether there were genetically based differences in timing of maturation between the two populations (i.e. differences between 'pure' crosses) and whether this divergence is likely to be caused by additive genetic effects (i.e. reciprocal 'hybrid' means are both equal and intermediate compared to 'pure' crosses) or maternal and/or nonadditive genetic effects (i.e. reciprocal 'hybrid' means deviate from the expected intermediacy). For example, under dominant gene action, the two reciprocal 'hybrid' crosses would be expected to be equal, but more similar to one of the 'pure' crosses, whilst if maternal effects were important, reciprocal 'hybrids' would be closer to their mothers' 'pure' line. In addition, using molecular sexing methods, we also investigated differences in timing of maturation between the sexes within and among different crosses.

Materials and methods

Sampling, breeding and rearing

Adult nine-spined sticklebacks forming the parental generation were collected with seine nets and minnow traps before or during the early phase of reproductive season (late May–mid June 2010) from two populations of which one was a Baltic Sea (Helsinki; 60°12′09″N, 25°10′58″E) and the other was a pond population (Pyöreälampi; 66°15′40″N, 29°26′00″E). The marine site was a shallow bay with low salinity (ca. 6.0 psu; Shimada *et al.*, 2011), where nine-spined sticklebacks coexist with competitors (e.g. three-spined sticklebacks [*Gasterosteus aculeatus*]) and several sympatric piscine predators (e.g. pike [*Esox lucius*] and perch [*Perca fluviatilis*]). The pond site was a freshwater pond with less than 5-ha surface area, where nine-spined sticklebacks are the only fish species, apart from introduced whitefish (*Coregonus lavaretus*) which are now either extinct or present in very low density. Nine-spined sticklebacks from the Helsinki population are 'normal sized' (usually < 5 cm; Ab Ghani *et al.*, 2012) and can mature as early as a year after hatching (Herczeg *et al.*, 2009; Shimada *et al.*, 2011). Nine-spined sticklebacks from the Pyöreälampi population are 'giant sized' (5 + year old fish being ca. 8–10 cm and the largest fish we caught being > 11 cm), can live up to an age of at least 7 years and all mature fish aged were at least 2 years old (Herczeg *et al.*, 2009).

Between 14 and 20 June 2010, four types of crosses were produced with artificial fertilization of the wild-caught parents: two 'pure' crosses were created by crossing Helsinki males with Helsinki females (hereafter Hel Hel) or by crossing Pyöreälampi males with Pyöreälampi females (hereafter Pyö Pyö), and two reciprocal 'hybrid' crosses were created by crossing either Pyöreälampi males with Helsinki females (hereafter Pyö Hel) or Helsinki males with Pyöreälampi females (hereafter Hel Pyö). Ten full-sib families for each cross-type were produced (i.e. a total of 40 families). The artificial fertilizations (crosses) were made between randomly chosen males and females by gently squeezing the eggs out from the ripe females and pouring the sperm solution onto the eggs. Sperm solution was obtained by mincing the testicles of males over-anaesthetized with an overdose (ca. 100 mg L^{-1}) of MS-222. Fertilizations were made on the sampling site and in the laboratory. Thus, conditions for the fertilized eggs were not fully identical for the first 2 days of egg development, but we have shown earlier that this difference does not influence the subsequent egg size (Ab Ghani *et al.*, 2012). Clutches were kept in petri dishes in filtered tap water (water changed twice a day) until hatched. Developing clutches were checked under a dissecting microscope regularly and dead or unfertilized eggs were removed.

After the hatched larvae started to swim freely (ca. 7 days after hatching), five fish per family per cross-type were randomly individualized to 1.4-L tanks in four Allentown Zebrafish Racks Systems (hereafter rack, Aquaneering Inc., San Diego, CA, USA). Each rack contained 100 units of 1.4-L tanks and had a closed water circulation system with UV, physical, chemical and biological filtering. Visual contact between the tanks was blocked by white plastic panels. Fish were reared in freshwater (salinity 0 psu) and fed *ad libitum* with live brine shrimp (*Artemia* sp.) nauplii for the first two months and thereafter with frozen bloodworms. They were kept in 14 : 10 hours light : dark photoperiod at 17 °C water temperature throughout the experiment. Three hundred days after hatching, all fish were subjected to artificial hibernation which was accomplished by gradually lowering the water temperature to 4 °C and shifting the

photoperiod to 0 : 24 hours light : dark within 2 weeks. This artificial hibernation lasted for 30 days. After that, over a 2-week period, water temperature was gradually increased back to 17 °C and the photoperiod was gradually switched to 24 : 0 hours light : dark to stimulate testes and ovary development (Baggerman, 1985). Since the beginning of sexual maturation can be influenced by abiotic variables (e.g. temperature: Garrard *et al.*, 1974; Marion, 1982; Kuparinen *et al.*, 2011; photoperiod: Baggerman, 1985; Taranger *et al.*, 1999), every effort was made to standardize conditions under which the experimental fish were raised, so that differences or similarities in age of gonad maturation could not be attributed to variation in rearing conditions.

Data collection

To record the timing of maturation, each individual fish was visually inspected every day starting from the day when the artificial hibernation ended. At this time point, a total of 187 individuals were recorded (Hel–Hel: 44, Hel–Pyö: 48, Pyö–Hel: 49 and Pyö–Pyö: 46) and all fish were ca. 344 days old. Sexually mature males develop white pelvic spines and black body colouration, while sexually mature females develop a protruding belly as a consequence of ovaries containing ripe eggs. Based on these unambiguous visual criteria, the timing of maturation was observed for a period of 8 weeks by the same person (N.I. Ab Ghani). At the end of the observation period, immature individuals were marked as censored. This was done because their maturation date remained unknown (right-censoring) and biases might ensue unless this is accounted for (Leung *et al.*, 1997). Hence, our data set included both matured and immature individuals. We identified the sex of the immature individuals using a molecular sexing method following Shikano *et al.* (2011). In short, DNA was extracted from a small portion of caudal fins of the immature individuals using the chelex method (Walsh *et al.*, 1991). Polymerase chain reactions (PCRs) were carried out with primers amplifying a sex-linked microsatellite marker Stn19 (Shikano *et al.*, 2011). The PCRs contained 2 × buffer solutions, 0.6 × $MgCl_2$, 0.16 × dNTPs, 0.1 × Taq polymerase, 2 *ρ*mol of each primer, approximately 10–20 ng of template DNA and MQ water for a final volume of 20 *μ*L. The PCRs were performed with the following cycle: an initial activation step at 95 °C for 3 min, followed by 30 s at 95 °C, 30 s at 53 °C and 30 s at 72 °C for 30 cycles, and final extension at 72 °C for 5 min. PCR products were visualized on 2% agarose gel electrophoresis and run for 90 min. Males were identified by the presence of 174 and/or 176 alleles, whereas females were identified by the absence of these alleles and the presence of 158 and/or 160 alleles (Shikano *et al.*, 2011).

Statistical analyses

Data were analysed at two levels. First, we were interested in the probability of maturation during the 8-week observation period. Because our data set contained family structure (random effect) and immature individuals (censored) after the last observation day (ca. 400 days old), we used the Survival Kit v6 software (Ducrocq *et al.*, 2010) which is able to handle mixed-model survival analyses with random effects and censored data. The probability of maturation was modelled in Cox regression with origin of father, origin of mother, sex and their interactions as predictors. Family was treated as a random factor. For a finer resolution, we also ran pairwise Cox regressions between different crosses, separately for the two sexes (as sex was a strong predictor, see Results). As these pairwise Cox regressions were not independent, the significance of the results was evaluated after false discovery rate (FDR) adjustment (Benjamini & Hochberg, 1995). To further explore the sex differences in timing of maturation, generalized linear mixed model (GLMM) analyses were performed for (a) all individuals (i.e. mature and immature) and (b) matured individuals only. Here, the sex was considered a binomial response variable (applying logit-link function) to test if there were any sex-bias in (a) maturation or (b) in general among the cross-types. Here, cross-type was included as a fixed effect and family within cross-type as a random effect. The GLMMs were run using the SAS 9.2 (SAS Institute Inc, 2007) software package.

Results

By the end of the observation period, all the 'pure' Helsinki (Hel–Hel) fish had matured, whereas only 39% of 'pure' Pyöreälampi (Pyö–Pyö) fish had matured (90% of males and none of females; Fig. 1a,b). Individuals from both of the 'hybrid' crosses matured (98% of 'hybrid' Hel–Pyö and 96% of 'hybrid' Pyö–Hel) as early as the 'pure' Helsinki fish (Fig. 1a,b). Cox regression analyses of the probability of maturation among the four types of crosses during the experimental period showed strong effects of mother origin ($\chi^2 = 42.05$, d.f. = 1, $P < 0.0001$), father origin ($\chi^2 = 28.12$, d.f. = 1, $P < 0.0001$) and mother origin × father origin interaction ($\chi^2 = 61.13$, d.f. = 3, $P < 0.0001$). This suggests that the differences in timing of maturation between populations were due to a combination of additive and nonadditive genetic effects, supporting the genetic basis of the population divergence. The fact that both 'hybrid' crosses converged towards the maturation pattern of the Helsinki fish, rather than towards the patterns in population of their maternal origin (Fig. 1a,b), indicates that variation in maturation time was caused by nonadditive genetic rather than maternal effects. We also found strong effects of sex ($\chi^2 = 58.18$, d.f. = 1, $P < 0.0001$), with males maturing earlier than females (Fig. 1a,b). Sex × mother origin ($\chi^2 = 5.50$, d.f. = 3, $P = 0.14$), sex × father origin ($\chi^2 = 4.57$, d.f. = 3, $P = 0.21$) and sex × mother origin × father origin ($\chi^2 = 2.34$, d.f. = 6, $P = 0.89$) interactions were all nonsignificant, suggesting similar pattern of sexual asynchrony in maturation in both populations (Fig. 1a,b). The pairwise Cox regressions further strengthened the inference: (a) the 'pure' crosses differed from each other (irrespective of sex, all $P < 0.0001$), (b) the 'hybrid' crosses did not differ from each other (irrespective of sex, all $P > 0.62$), (c) the 'hybrid' crosses differed from the 'pure' Pyöreälampi cross (irrespective of sex, all $P < 0.0001$) but (d) did not differ from the 'pure' Helsinki cross (irrespective of sex, all $P > 0.08$; Table 1).

The sex ratios of matured individuals by the end of observation period were even across families ($\chi^2 = 35.79$, d.f. = 36, $P = 0.43$), but heterogeneous across the different cross-types ($\chi^2 = 30.30$, d.f. = 3, $P < 0.001$). This heterogeneity was derived mainly from the fact that none of the females in Pyö–Pyö crosses matured (Table 2). However, also the sex-ratio in Hel–Pyö cross deviated significantly from the expected 1 : 1 ratio at the end of the observation period (Table 2). This deviation, however, did not owe to sex differences in maturation probability as there was a sex-ratio bias in this particular cross-type at the outset (Table 2).

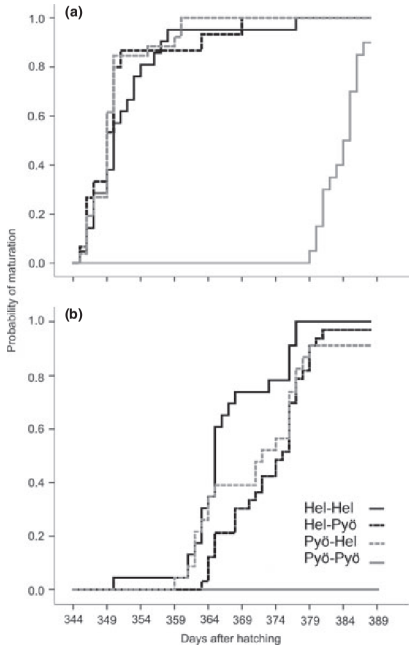


Fig. 1 Probability of maturation in the 'pure' and 'hybrid' crosses of nine-spined stickleback (a) males and (b) females within 8 weeks after hibernation. 'Hel' denotes Helsinki (Baltic Sea, marine) parents and 'Pyö' denotes Pyöreälampi (pond) parents. The origin of the father is first and the origin of mother second.

Table 1 Pairwise comparison of timing of maturation in different nine-spined stickleback cross-types (and sexes) based on Cox regression survival analyses.

	d.f.	χ^2	*P*
Both sexes			
Hel–Hel: Pyö–Pyö	**1**	**75.70**	**< 0.0001**
Hel–Hel: Hel–Pyö	1	4.81	0.028
Hel–Hel: Pyö–Hel	1	0.36	0.55
Hel–Pyö: Pyö–Hel	1	1.56	0.21
Pyö–Pyö: Hel–Pyö	**1**	**21.90**	**< 0.0001**
Pyö–Pyö: Pyö–Hel	**1**	**58.23**	**< 0.0001**
Males			
Hel–Hel: Pyö–Pyö	**1**	**31.19**	**< 0.0001**
Hel–Hel: Hel–Pyö	1	0.22	0.64
Hel–Hel: Pyö–Hel	1	0.30	0.58
Hel–Pyö: Pyö–Hel	1	0.09	0.77
Pyö–Pyö: Hel–Pyö	**1**	**35.29**	**< 0.0001**
Pyö–Pyö: Pyö–Hel	**1**	**40.91**	**< 0.0001**
Females			
Hel–Hel: Pyö–Pyö	**1**	**51.54**	**< 0.0001**
Hel–Hel: Hel–Pyö	1	3.02	0.082
Hel–Hel: Pyö–Hel	1	1.00	0.32
Hel–Pyö: Pyö–Hel	1	0.24	0.62
Pyö–Pyö: Hel–Pyö	**1**	**51.99**	**< 0.0001**
Pyö–Pyö: Pyö–Hel	**1**	**42.51**	**< 0.0001**

Hel, Helsinki; Pyö, Pyöreälampi. The origin of the father is first and the origin of mother second. Comparisons significant after FDR adjustment in boldface.

Discussion

The most salient findings of this study were the clear, genetically based population differences in timing of maturation among two stickleback populations, as well as the evidence for a dominant mode of gene action underlying this divergence. The fact that whenever either of the parents originated from the marine population, the offspring matured as early as the offspring from the 'pure' marine population cross, indicates that the marine population (the ancestral) may be carrying dominant allele(s) determining early maturation. By inference, the pond population (the descendant) may have been subjected to natural selection against these dominant allele(s) resulting in the accumulation of recessive allele(s) determining delayed maturation. This type of dominant-recessive allele patterns has

Table 2 Sex ratios (SR) and tests for deviations from the expected 1 : 1 SR in different nine-spined stickleback cross-types.

	Matured individuals					All individuals				
Cross-type	Males	Females	SR	χ^2	*P*	Males	Females	SR	χ^2	*P*
Hel–Hel	21	23	0.91	0.09	0.763	21	23	0.91	0.09	0.763
Hel–Pyö	15	32	0.47	6.15	0.013	15	33	0.45	6.75	0.009
Pyö–Hel	26	21	1.24	0.53	0.466	26	23	1.13	0.18	0.668
Pyö–Pyö	18	0	∞	24.09	< 0.001	20	26	0.77	0.78	0.376

Hel, Helsinki; Pyö, Pyöreälampi. The origin of the father is first and the origin of mother second.

previously been reported in platyfish (*Xiphophorus maculatus*), where three populations were reared in a laboratory for 35 years (Kallman *et al.*, 1973; Kallman & Borkoski, 1978). In platyfish, five alleles at the pituitary (*P*) locus have been detected to cause variation in timing of maturation: the dominant '*P1*' allele causes early maturation whilst the recessive '*P5*' allele causes delayed maturation (Kallman & Borkoski, 1978). In contrast, no evidence for any dominant mode of inheritance in timing of maturation amongst three rainbow trout (*Oncorhynchus mykiss*) strains subject to commercial selection was found (Quinton *et al.*, 2004). In Chinook salmon (*O. tshawytscha*), strong evidence for additive genetic divergence in timing of maturation was found between two populations, and this divergence was hypothesized to reflect adaptation to different spawning environments rather than genetic drift or founder effects (Quinn *et al.*, 2000). To our knowledge, no other studies on timing of maturation using reciprocal crosses between diverged populations have been published. Hence, our findings provide rare evidence for genetically based divergence in timing of maturation among wild living vertebrate populations.

We acknowledge that the observed differences in timing of maturation between the marine and the pond populations of nine-spined sticklebacks might also be influenced by cross-generational environmental effects (e.g. Rossiter, 1996; Mousseau & Fox, 1998). Although all the fish were reared under identical environmental conditions, we only had F_1 fish whose parents originated from the wild, and carryover effects are possible (Rossiter, 1996; Lynch & Walsh, 1998). However, given that we found no evidence for maternal effects, and the results from a previous study using the same populations are concordant with ours (Shimada *et al.*, 2011), it seems unlikely that carry-over effects would have exerted a strong influence on the results. Furthermore, timing of maturation in the marine population has been shown to be heritable (h^2 = 0.15–0.26; Shimada *et al.*, 2011), suggesting presence of additive genetic variance in this trait, and thereby, potential for evolutionary divergence.

As to the potential selective factors explaining the divergence in timing of maturation between the marine and the pond populations, we note that one of the main differences between the two populations is the complete lack of piscine predation in the pond population and the possibly consequent increased adult survival rates. This together with the increased importance of intraspecific competition in the pond habitat has been hypothesized to be the driver of divergent life histories in marine and pond populations (e.g. Herczeg *et al.*, 2009, 2010, 2012). In guppies (*Poecilia reticulata*), fish from high-piscine predation environments evolve towards earlier maturity and reduced body size due to high adult mortality rates, whereas guppies from low-predation environments evolve towards later maturity and increased body size due to low adult mortality and birth rates (Reznick & Ghalambor, 2005). Natural selection on body size and growth rate are other factors that may affect the timing of maturation and explain the observed differences in timing of maturation between the populations (e.g. Stearns & Koella, 1986; Morita & Fukuwaka, 2006). The maturation reaction norm explains that maturation should be flexible along an age-size trajectory and as a function of both environmental and genetic (i.e. evolution) effects. In the nature of life-history traits – such as timing of maturation – is that the traits are often (genetically) correlated with other traits: when selection is applied to one trait, changes may occur also in correlated traits. Thus, a delay in maturity may decrease growth rates depending on whether growth rates are associated with adult or juvenile (or both) mortality rates, and whether body size–fecundity relationship owes mainly to environment or genetic effects (Dieckmann & Heino, 2007). For instance, when population density is reduced due to high adult mortality rates as a consequence of high predation, competition is reduced and resource availability increases and natural selection might favour increased growth rates leading to evolution of early maturation at small body size. We have found that timing of maturation in males was correlated with their asymptotic body size – but not with their growth rate in these data (N.I. Ab Ghani, A. Kuparinen, T. Leinonen & J. Merilä, unpublished). This suggests that at least in males, but possibly also in females, the observed differences in timing of maturation between the populations may result from differential selection acting on the body size–fecundity relationship. Individuals in marine

populations are subject to heavier predation pressure by piscine predators, which is expected to lead high adult mortality rates and reduced competition and hence, result in selection for earlier maturation at a smaller size (e.g. Jennions *et al.*, 2005; Reznick & Ghalambor, 2005).

Fisheries-induced changes in maturation reaction norms are common (Dieckmann & Heino, 2007). Some part of changes in timing of maturation due to fisheries might have a genetic component, but the relative importance of environmental and genetic influences on maturation in this context remains uncertain (Kuparinen & Merilä, 2007; Law, 2007). The finding that recessive alleles determine delayed maturation sheds light on problems related to fisheries-induced evolution and recovery of fish stocks. Many commercially exploited fish populations appear to have evolved towards earlier maturation (Law & Grey, 1989; Law, 2000; Kuparinen & Merilä, 2007) – a trend that has been found to be difficult to reverse after closing the fisheries (Law & Grey, 1989; Enberg *et al.*, 2009). This inability of the fish stocks to recover and reverse the trend back towards later maturation would be reinforced by the recessive nature of the alleles responsible for delayed maturation. After being driven to low frequency (if not eliminated) by the fisheries, reversal towards delayed maturation would be slow to occur because selection on rare recessives is not very efficient (Hartl & Clark, 2007). This is because almost all individuals with the rare allele are expected to be heterozygous, and the heterozygous individuals for the recessive allele would have the same phenotype (maturation time) and fitness as homozygotes for the dominant allele (Hartl & Clark, 2007). In other words, the trend towards earlier maturation would be expected to be more swiftly reversed if the delayed maturation was a dominant trait.

We also found strong and consistent sexual asynchrony in timing of maturation in all cross-types – males matured earlier than females. In the extreme case of the pond–pond cross, none of the females matured during our observation period. These findings are in line with those of Shimada *et al.* (2011), who also observed no maturation among pond females (140 days after hatching). Early maturation of males makes sense in the light of the fact that the reproductive output of females is likely to be more strongly influenced by their size than that of males (Roff, 1992), and timing of maturation in most species, including the nine-spined stickleback, is associated with cessation of growth (Shimada *et al.*, 2011). Previous studies have reported that selection on females is likely a driver of body size and growth rate divergence between these two populations (Herczeg *et al.*, 2010, 2012). This pattern is also commonly observed in many other animals and plants (Fagerström & Wiklund, 1982; Ni & Sandeman, 1984; Myers *et al.*, 1986; Donaldson & Benfey, 1987; Simmons & Johnston, 1997; Calabrese & Fagan, 2004). Hence, this may also suggests that the observed evolutionary divergence in timing of maturation between these two nine-spined stickleback populations is driven by selection acting on females. That said, we cannot dismiss the possibility that males derive benefits from protandry, and that there are population differences in the strength of selection favouring early maturation in males.

Finally, we acknowledge the two distinct limitations of this study: lack of replication in respect to the number of populations used and lack of back-cross design, which would have allowed explicit dissection of dominance, additive and maternal effect contributions to the population divergence (e.g. Lynch & Walsh, 1998). As to the population replication, logistic constraints prohibited larger sized experiment based on individual rearing. Within these logistic constraints, we preferred accurate individual level data on maturation from fewer populations to a less accurate and smaller data set based on multiple population approach. In fact, our original aim was to continue this experiment to F_2-generation by back-crossing the hybrids to their parental stocks, but as none of the females in the pond-cross matured, these plans were abandoned. However, as no evidence for large maternal effects were found in this study, it seems unlikely that the back-cross study would have lead to any discoveries fundamentally different from what was uncovered here. Yet, the relative sizes of dominance and additive genetic contributions to population divergence in timing of maturation remain to be estimated in future studies.

In summary, our results suggest a genetic basis for differences in timing of maturation between two divergent nine-spined stickleback populations and that the timing of maturation is inherited in a dominant-recessive fashion. The low-predation pond population (the descendent) has likely been subjected to strong selective forces against dominant alleles (early maturation) resulting in the evolution of delayed maturation. Hence, our study presents a step towards better understanding the genetic underpinnings and evolution of timing of maturation, and considering the genetic resources available for stickleback research (e.g. Peichel *et al.*, 2001; Shapiro *et al.*, 2009; Shikano *et al.*, 2010b), the nine-spined stickleback system provides a good candidate system for future studies aiming to uncover the genes underlying variation in timing of maturation.

Acknowledgments

Thanks are due to Marika Hölttä, Niina Nurmi, Mirva Turtianen and Linda Uoti for their help during the experiments. Special thanks to Anna Kuparinen, John Loehr and two anonymous reviewers for their comments on an earlier version of this manuscript, and Gábor Mészáros for his advice on running the Survival

Kit. Our work was supported by the Academy of Finland (grants #129662, #134728 and #218343 to JM and #128716 for GH) and Universiti Putra Malaysia (to IAG). The research leading to these results has also received funding from the European Community's Seventh Framework Programme (FP/2007-2013) under grant agreement n217246 made with BONUS, the joint Baltic Sea research and development programme (to JM). The experiment was conducted under the license from the Finnish National Animal Experiment Board (License no. STH211A). The authors declare that they have no conflict of interest.

References

Ab Ghani, N.I., Herczeg, G. & Merilä, J. 2012. Body size divergence in nine-spined sticklebacks: disentangling additive genetic and maternal effects. *Biol. J. Linn. Soc.* **107**: 521–528.

Baggerman, B. 1985. The roles of daily and annual biological rhythms in the photoperiodic regulation of the breeding season in the stickleback *Gasterosteus aculeatus* L. *Behaviour* **93**: 1–7.

Benjamini, Y. & Hochberg, Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Statist. Soc. B* **57**: 289–300.

Berven, K.A. 1982. The genetic basis of altitudinal variation in the wood frog *Rana sylvatica* I. An experimental analysis of life history traits. *Evolution* **36**: 962–983.

Bruneaux, M., Johnston, S.E., Herczeg, G., Merilä, J., Primmer, C.R. & Vasemägi, A. 2012. Molecular evolutionary and population genomic analysis of the nine-spined stickleback using a modified restriction-site-associated DNA tag approach. *Mol. Ecol.* **22**: 565–582.

Calabrese, J.M. & Fagan, W.F. 2004. Lost in time, lonely, and single: reproductive asynchrony and the allele effect. *Am. Nat.* **164**: 25–37.

Campton, D.E. & Gall, G.A.E. 1988. Responses to selection for body size and age at sexual maturity in the mosquitofish, *Gambusia affinis*. *Aquaculture* **68**: 221–241.

Charpentier, M.J.E., Tung, J., Altmann, J. & Alberts, S.C. 2008. Age at maturity in wild baboons: genetic, environmental and demographic influences. *Mol. Ecol.* **17**: 2026–2040.

Dieckmann, U. & Heino, M. 2007. Probabilistic maturation reaction norms: their history, strengths, and limitations. *Mar. Ecol. Prog. Ser.* **335**: 253–269.

Donaldson, E.M. & Benfey, T.J. 1987. Current status of sex manipulation. In: *Proceedings of Third Intlernational Symposium on Reproductive Physiology of Fish.* (D.R. Idler & L. Crim, eds), pp. 108–119. Memorial University Press, St. John's Newfoundland, Canada.

Ducrocq, V., Sölkner, J. & Mészáros, G. 2010. Survival Kit v6 - A software package for survival analysis. In: 9th World Congress on Genetics to Livestock Production, August 1-6, 2010. Leipzig, Germany. http://www.nas.boku.ac.at/1897.html

Enberg, K., Jørgensen, C., Dunlop, E.S., Heino, M. & Dieckmann, U. 2009. Implications of fisheries-induced evolution for stock rebuilding and recovery. *Evol. Appl.* **2**: 394–414.

Fagerström, T. & Wiklund, C. 1982. Why do males emerge before females? Protandry as a mating strategy in male and female butterflies. *Oecologia* **52**: 164–166.

Gadgil, M. & Bossert, W.H. 1970. Life historical consequences of natural selection. *Am. Nat.* **104**: 1–24.

Garrard, G., Harrison, G.A. & Weiner, J.S. 1974. Genetic and environmental factors determining the morphological maturation of the mouse and its relationship with weight growth. *J. Embryol. Exp. Morph.* **31**: 247–261.

Gibbons, J.W., Semlitsch, R., Greene, J. & Schubauer, J.P. 1981. Variation in age and size at maturity of the Slider turtle (*Pseudemys scripta*). *Am. Nat.* **117**: 841–845.

Hartl, D.L. & Clark, A.G. 2007. *Principles of Population Genetics*, 4th edn. Sinauer Associates, Sunderland, MA.

Heibo, E. 2003. *Life-history Variation and Age at Maturity in Eurasian Perch (Perca fluviatilis L.)*. PhD thesis. Swedish University of Agricultural Sciences, Umeå.

Herczeg, G., Gonda, A. & Merilä, J. 2009. Evolution of gigantism in nine-spined sticklebacks. *Evolution* **63**: 7–27.

Herczeg, G., Gonda, A. & Merilä, J. 2010. Rensch's rule inverted – female-driven gigantism in nine-spined stickleback *Pungitius pungitius*. *J. Anim. Ecol.* **79**: 581–588.

Herczeg, G., Gonda, A., Kuparinen, A. & Merilä, J. 2012. Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? *Evol. Ecol.* **26**: 109–122.

Hernaman, V. & Munday, P.L. 2005. Life-history characteristics of coral reef gobies II. Mortality rate, mating system and timing of maturation. *Mar. Ecol. Prog. Ser.* **290**: 223–237.

Houle, D. & Rowe, L. 2003. Natural selection in a bottle. *Am. Nat.* **161**: 50–67.

Jennions, M.D., Wong, B.B.M., Cowling, A. & Donnelly, C. 2005. Life-history phenotypes in a live-bearing fish *Brachyrhaphis episcopi* living under different predator regimes: seasonal effects? *Environ. Biol. Fish.* **76**: 211–219.

Kallman, K.D. & Borkoski, V. 1978. A sex-linked gene controlling the onset of sexual maturation in female and male platyfish (*Xiphophorus maculatus*), fecundity in females and adult size in males. *Genetics* **89**: 79–119.

Kallman, K.D., Schreibman, M.P. & Borkoski, V. 1973. Genetic control of gonadotrop differentiation in the platyfish, *Xiphophorus maculatus* (Poeciliidae). *Science* **58**: 678–680.

Kolluru, G.R. & Reznick, D.N. 1996. Genetic and social control of male maturation in *Phallichthys quadripunctatus* (Pisces: Poeciliidae). *J. Evol. Biol.* **9**: 695–715.

Kuparinen, A. & Merilä, J. 2007. Detecting and managing fisheries-induced evolution. *Trends Ecol. Evol.* **22**: 652–659.

Kuparinen, A., Cano, A.J.M., Loehr, J., Herczeg, G., Gonda, A. & Merilä, J. 2011. Fish age at maturation is influenced by temperature independently of growth. *Oecologia* **167**: 435–443.

Law, R. 2000. Fishing, selection, and phenotypic evolution. *ICES J. Mar. Sci.* **57**: 659–668.

Law, R. 2007. Fisheries-induced evolution: present status and future directions. *Mar. Ecol. Prog. Ser.* **335**: 271–277.

Law, R. & Grey, D.R. 1989. Evolution of yields from populations with age-specific cropping. *Evol. Ecol.* **3**: 343–359.

Leung, K.-M., Elashoff, R.M. & Afifi, A.A. 1997. Censoring issues in survival analysis. *Annu. Rev. Public Health* **18**: 83–104.

Lynch, M. & Walsh, B. 1998. *Genetics and Analysis of Quantitative Traits*. Sinauer Associates, Sunderland, MA.

Marion, K.R. 1982. Reproductive cues for gonadal development in temperate reptiles: temperature and photoperiod effects on the testicular cycle of the lizard *Sceloporus undula*. *Herpetologica* **38**: 26–39.

Mollet, F.M., Kraak, S.B.M. & Rijnsdorp, A.D. 2007. Fisheries-induced evolutionary changes in maturation reaction norms in North Sea sole *Solea solea*. *Mar. Ecol. Prog. Ser.* **351**: 189–199.

Morita, K. & Fukuwaka, M. 2006. Does size matter most? The effect of growth history on probabilistic reaction norm for salmon maturation. *Evolution* **60**: 1516–1521.

Mousseau, T.A. & Fox, C.W. 1998. *Maternal Effects as Adaptations*. Oxford University Press, New York.

Myers, R.A., Hutchings, J.A. & Gibson, R.J. 1986. Variation in male parr maturation within and among populations of Atlantic salmon, *Salmo salar*. *Can. J. Fish. Aquat. Sci.* **43**: 1242–1248.

Ni, I.H. & Sandeman, E.J. 1984. Size at maturity for northwest Atlantic redfishes (Sebastes). *Can. J. Fish. Aquat. Sci.* **41**: 1753–1762.

Peichel, C.L., Nereng, K.S., Ohgi, K.A., Cole, B.L.E., Colosimo, P.F., Buerkle, C.A. *et al.* 2001. The genetic architecture of divergence between threespine stickleback species. *Nature* **414**: 901–905.

Quinn, T.P., Unwin, M.J. & Kinnison, M.T. 2000. Evolution of temporal isolation in the wild: genetic divergence in timing of migration and breeding by introduced Chinook salmon populations. *Evolution* **54**: 1372–1385.

Quinton, C.D., McKay, L.R. & McMillan, I. 2004. Strain and maturation effects on female spawning time in diallele crosses of three strains of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **234**: 99–100.

Reznick, D.N. & Ghalambor, C.K. 2005. Can commercial fishing cause evolution? Answers from guppies (*Poecilia reticulata*). *Can. J. Fish. Aquat. Sci.* **62**: 791–801.

Reznick, D., Bryga, H. & Endler, J.A. 1990. Experimentally induced life-history evolution in a natural population. *Nature* **346**: 357–359.

Rijnsdorp, A.D. 1993. Fisheries as a large-scale experiment on life-history evolution: disentangling phenotypic and genetic effects in changes in maturation and reproduction of North Sea plaice, *Pleuronectes platessa* L. *Oecologia* **96**: 391–401.

Roff, D.A. 1992. *The Evolution of Life Histories: Theory and Analysis*. Chapman & Hall, New York.

Roff, D.A. 2002. *Life History Evolution*. Sinauer Associates, Sunderland, MA.

Rossiter, M. 1996. Incidence and consequences of inherited environmental effects. *Annu. Rev. Ecol. Sys.* **27**: 451–476.

SAS Institute Inc. 2007. *SAS/STAT (R) User's Guide*, 2nd edn. SAS Institute Inc, Cary, NC.

Shapiro, M.D., Summers, B.R., Balabhadra, S., Aldenhoven, J.T., Miller, A.L., Cunningham, C.B. *et al.* 2009. The genetic architecture of skeletal convergence and sex determination in ninespine sticklebacks. *Curr. Biol.* **19**: 1140–1145.

Shikano, T., Shimada, Y., Herczeg, G. & Merilä, J. 2010a. History *vs.* habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. *Mol. Ecol.* **19**: 1147–1161.

Shikano, T., Ramadevi, J., Shimada, Y. & Merilä, J. 2010b. Utility of sequenced genomes for microsatellite marker development in non-model organisms: a case study of functionally important genes in nine-spined sticklebacks (*Pungitius pungitius*). *BMC Genomics* **11**: 334–347.

Shikano, T., Herczeg, G. & Merilä, J. 2011. Molecular sexing and population genetic inference using a sex-linked microsatellite marker in the nine-spined stickleback (*Pungitius pungitius*). *BMC Res. Notes* **4**: 119–124.

Shimada, Y., Shikano, T., Kuparinen, A., Gonda, A., Leinonen, T. & Merilä, J. 2011. Quantitative genetics of body size and timing of maturation in two-nine-spined stickleback (*Pungitius pungitius*) populations. *PLoS ONE* **6**: e28859.

Simmons, A.M. & Johnston, M.O. 1997. Developmental instability as a bet-hedging strategy. *Oikos* **80**: 401–406.

Stearns, S.C. 1984. Heritability estimates for age and length at maturity in two populations of mosquito fish that shared ancestors in 1905. *Evolution* **38**: 368–375.

Stearns, S.C. 1992. *The Evolution of Life Histories*. Oxford University Press, New York.

Stearns, S.C. 2000. Life history evolution: successes, limitations, and prospects. *Naturwissenschaften* **87**: 476–486.

Stearns, S.C. & Koella, J.C. 1986. The evolution of phenotypic plasticity in life-history traits: prediction of reaction norms for age and size at maturity. *Evolution* **40**: 893–913.

Taranger, G.L., Haux, C., Hansen, T., Stefansson, S.O., Björnsson, B.T., Walther, B.T. *et al.* 1999. Mechanisms underlying photoperiodic effects on age at sexual maturity in Atlantic salmon, *Salmo salar*. *Aquaculture* **177**: 47–60.

Walsh, P.S., Metzger, D.A. & Higuchi, R. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* **10**: 506–513.

Wright, P.J. 2007. Understanding the maturation process for field investigations of fisheries-induced evolution. *Mar. Ecol. Prog. Ser.* **335**: 279–283

Data deposited at Dryad: doi: 10.5061/dryad.f8g39

Received 5 September 2012; revised 6 November 2012; accepted 23 November 2012

Journal of FISH BIOLOGY
Published for the Fisheries Society of the British Isles

Journal of Fish Biology (2013) **82**, 318–331
doi:10.1111/jfb.12006, available online at wileyonlinelibrary.com

Optimal growth strategies under divergent predation pressure

S. Aikio*†, G. Herczeg‡, A. Kuparinen‡ and J. Merilä‡

**Plant Ecology and Population Biology Research Group, Department of Biology, P. O. Box 3000, FIN-90014 University of Oulu, Oulu, Finland and ‡Ecological Genetics Research Unit, Department of Biosciences, P. O. Box 65, FIN-00014 University of Helsinki, Helsinki, Finland*

(Received 4 April 2012, Accepted 19 October 2012)

The conditions leading to gigantism in nine-spined sticklebacks *Pungitius pungitius* were analysed by modelling fish growth with the von Bertalanffy model searching for the optimal strategy when the model's growth constant and asymptotic fish size parameters are negatively related to each other. Predator-related mortality was modelled through the increased risk of death during active foraging. The model was parameterized with empirical growth data of fish from four different populations and analysed for optimal growth strategy at different mortality levels. The growth constant and asymptotic fish size were negatively related in most populations. Optimal fish size, fitness and life span decreased with predator-induced mortality. At low mortality, the fitness of pond populations was higher than that of sea populations. The differences disappeared at intermediate mortalities, and sea populations had slightly higher fitness at extremely high mortalities. In the scenario where all populations mature at the same age, the pond populations perform better at low mortalities and the sea populations at high mortalities. It is concluded that a trade-off between growth constant and asymptotic fish size, together with different mortality rates, can explain a significant proportion of body size differentiation between populations. In the present case, it is a sufficient explanation of gigantism in pond *P. pungitius*.

Key words: body size; gigantism; model; population; *Pungitius*.

INTRODUCTION

Given its importance to fitness, body size is a trait of particular ecological and evolutionary significance (Peters, 1983; Roff, 1992; Stearns, 1992). In many species, both sexual (Shine, 1989; Andersson, 1994) and fecundity selection favours large body size (Arendt, 2010). Furthermore, by providing a size refuge from size-limited predators that are restricted to smaller prey items, predation may also select for large body size (Werner & Gilliam, 1984; Abrams & Rowe, 1996; Urban, 2008).

Despite the benefits of a larger body size, individuals of most natural populations do not seem to evolve to a consistently larger size (Calow, 1982; Atchley, 1984; Blanckenhorn, 2000). This is most probably due to the fact that large adult body size is in a trade-off with growth constant (*i.e.* the rate at which the body size at maturation is reached, often estimated with the *k* parameter from the von Bertalanffy equation; von Bertalanffy, 1938), so individuals that approach the estimated final size quickly are predicted to remain smaller than their slower growing conspecifics (Berrigan & Charnov, 1994). Fast growth, in terms of added body volume per time, can be advantageous if it allows individuals to reach reproductive size at a younger age, and, thus, increasing the time available for reproduction. On the other hand, small body size can reduce access to resources and mates (Sibly & Calow, 1986; Blanckenhorn & Demont, 2004). Size-unlimited predation can, however, be one particular factor selecting against large body size and fast growth: predators might actively select larger individuals as well as fast growing individuals, which are more active foragers as they have to fulfil their elevated energy needs (Lima & Dill, 1990; Abrams & Rowe, 1996; Blanckenhorn, 2000; Day & Rowe, 2002; Biro *et al*., 2004, 2006). Surprisingly, while the evolutionary and ontogenetic mechanisms behind size-limited predation have been studied in some detail, studies focusing on population divergence in growth strategies induced by variation in size-unlimited predation are scarce (Dmitriew, 2010).

Nine-spined stickleback *Pungitius pungitius* (L. 1758) offers a good model to study optimal growth when selection pressures for body size differ. It is a small-sized (typically 4–5 cm in total length, L_T) teleost with a circumpolar distribution and occupies different habitats, from seas with large numbers of sympatric predatory fishes to isolated ponds where *P. pungitius* is the only fish species (Bănărescu & Paepke, 2001; Östlund-Nilsson *et al*., 2007). Repeated independent occurrence of gigantism (L_T >10 cm), as a combined result of higher year-to-year growth and extended longevity, has been reported from ponds (Kuusela, 2006; Herczeg *et al*., 2009*a*). Furthermore, in a comparison of fish from pond and sea populations reared in a common environment, a systematic divergence in growth strategies was found: pond fish had higher estimated final lengths than marine fish, while the latter had higher growth constants than the former (Herczeg *et al*., 2012) and continued their growth longer (Shimada *et al*., 2011). The proposed reason for these differences was habitat differences in the occurrence of size-unlimited predation. This idea has, however, not been rigorously analysed either empirically or theoretically.

In this study, the hypothesis that the negative correlation between growth constant and estimated asymptotic length (Charnov, 1993; Berrigan & Charnov, 1994), together with differing predation-related mortality may explain the marked body size divergence (Herczeg *et al*., 2009*a*, *b*), among *P. pungitius* populations was explored. More specifically, it was hypothesized that the lack of piscine predation and the consequent low mortality in pond environments make success in intraspecific competition (both in reproduction and resource monopolization) the key to increased fitness, and, thus, selection for extended growth and larger asymptotic size in ponds compared to habitats where predators are abundant and mortality is higher. To test this hypothesis, empirical data from common-garden pond-reared and sea *P. pungitius* were used to estimate the trade-off function between growth parameters within multiple populations. This trade-off function was then used to model the optimal growth in each population over a range of modelled predation-induced mortality rates. It was predicted that increased predation-induced mortality shifts the optimal growth strategy from slow growth constant and large asymptotic size towards fast growth and small asymptotic size.

†Author to whom correspondence should be addressed. Tel.: +358 29 448 1531; email: sami.aikio@gmail.com

MATERIALS AND METHODS

EXPERIMENTAL DATA

The experimental data used here is the same as described in Herczeg *et al.* (2009*a*, *b*, 2012). Adult *P. pungitius* were sampled from two coastal marine populations (Baltic Sea, Finland, and White Sea, Russia) with large numbers of sympatric predatory fish and two isolated ponds (Bynästjärnen, Sweden, and Pyöreälampi, Finland) where *P. pungitius* is the only fish species (Herczeg *et al.*, 2012). These four populations provided the parental stock for fish used in a common-garden experiment (Herczeg *et al.*, 2009*b*, 2012). The marine habitats had low salinity, as they were located in shallow coastal bays close to creek inlets, and the Baltic Sea is brackish water (Leppäranta & Myrberg, 2009). As described in detail in Herczeg *et al.* (2009*b*), five full-sib families from each population were produced *in vitro* and 10 randomly chosen offspring from each family (total $n = 200$) were reared individually in 1·4 l tanks contained in Allentown Zebrafish Rack Systems (Aquaneering Inc.; www.aquaneer.com). Temperature was set to 17° C and feeding was *ad libitum*. Fish were first photographed 4 days after hatching, before swimming freely and the first feeding. After the first photography on day 4, each individual fish was photographed at the following intervals: +1, +1, +2, +2, +3, +3, +4, +4, +8 and +8 weeks (= 36 weeks, 256 days). Photographs were taken with a digital camera (Panasonic DMC-FZ8; www.panasonic.com) mounted on a tripod. Photography was from outside the tanks; fish were put into a shallow layer of water and photographed laterally with a mm paper placed in each photograph for reference. Because of mortality (occurring mainly at early life stages) and fish removed (randomly) for other scientific purposes, the final growth data set included 86 fish. Of these, 21 were from the Baltic Sea (family representations: 6, 5, 4, 3 and 3), 23 from the White Sea (family representations: 7, 6, 5, 3 and 2), 20 from Bynästjärnen (family representations: 7, 4, 3, 3 and 3) and 22 from Pyöreälampi (family representations: 6, 5, 5, 5 and 1). Standard length (L_S; from the tip of the nose to the end of the tail base) was measured from the digital photographs by using TPSDig 2.10 (Rohlf, 2006) software. The experiments were conducted under licence number STH379A from the Finnish National Animal Experiment Board (ELLA).

GROWTH PARAMETERS

To describe the individual growth trajectories, von Bertalanffy growth curves were fitted to the measurements of L_S (mm) at age t (days), L_t, collected from each individual over time points given above (von Bertalanffy, 1938). The parameters of the curve were (units in parenthesis): L_∞ = asymptotic L_S (mm), L_0 = initial L_S (mm) at $t = 0$, k = growth constant (day^{-1}):

$$L_t = L_\infty - (L_\infty - L_0)e^{-kt}. \quad (1)$$

The growth constant, k, is a relative measure of growth that indicates how quickly fish length approaches L_∞ and it is linearly related to maximum growth rate on an absolute scale ($mm\ day^{-1}$; Appendix SI, Supporting Information). The growth model was fitted separately for each individual using a non-linear least-squares method resulting in 20–23 growth curves from each of the four populations (Table I). The von Bertalanffy model gave a very good fit to individual growth data (coefficient of determination ranges from $r^2 = 0{\cdot}977$ to 0·998). The average growth parameter values differed between the populations (Fig. 1 and Table I).

The values of k and L_∞ were negatively related to each other and the relationships differed between the populations (Fig. 2 and Table I). A negative exponential function was fitted separately to the parameter estimates L_∞ and k from each population and called trade-off function, where L_{int} is the length at the y-axis intercept corresponding to zero growth rate and λ is the steepness of the trade-off (Fig. 2 and Table I):

$$L_\infty = L_{int}\, e^{-\lambda k} \quad (2)$$

TABLE I. The number of individuals (N) and the parameter estimates (±95% C.I.) of the von Bertalanffy growth model (equation 1) for *Pungitius pungitius*: L_0, standard length (L_S) at the beginning; L_∞, asymptotic L_S; k, growth constant. The intercept L_{int} and steepness λ define the shape of the trade-off (equation 2; Fig. 2) between L_∞ and k in each population. The coefficient of determination (r^2) indicates the proportion of variation in L_∞ explained by the trade-off function. The maximum predator-induced mortality max m_p that fish have at unit fitness ($R_0 = 1$) is calculated separately for the two maturation scenarios: (1) all fish mature at the age of 1 year and (2) sea populations mature at 1 year and pond populations at 2 years of age

Parameter	Baltic Sea	White Sea	Bynästjärnen	Pyöreälampi
N	21	23	20	22
L_0 (mm)	5·19 ± 0·85	3·38 ± 0·00	5·03 ± 0·00	4·68 ± 0·00
L_∞ (mm)	48·9 ± 4·5	64·9 ± 4·9	85·8 ± 9·4	80·7 ± 10·4
k (day^{-1})	0·013 ± 0·002	0·013 ± 0·002	0·007 ± 0·001	0·010 ± 0·002
L_{int} (mm)	59·7 ± 18·1	106·9 ± 14·8	143·7 ± 14·6	155·2 ± 14·8
λ (day)	15·4 ± 24·1	39·7 ± 11·1	74·3 ± 14·8	66·1 ± 9·8
r^2	0·091	0·715	0·861	0·907
(1) max m_p	0·0673	0·0637	0·0669	0·0662
(2) max m_p	0·0673	0·0637	0·0601	0·0600

The trade-off function of each population determines the relationship between k and L_∞ parameters in subsequent modelling of the optimal phenotype. It is possible for the k and L_∞ parameters of the von Bertalanffy model to become negatively correlated as a result of a statistical artefact (Ratkowsky, 1986). The observed range of variation in these parameters

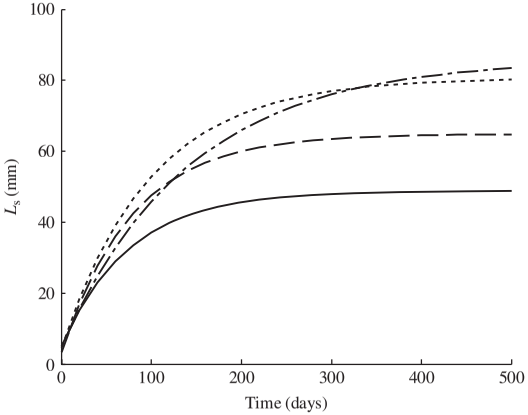


FIG. 1. The average von Bertalanffy growth function in terms of standard length (L_S) for the four different populations of *Pungitius pungitius*: Baltic Sea (——), White Sea (– –), Bynästjärnen (– · – ·) and Pyöreälampi (– – – –). The parameter estimates behind growth curves and the trade-off functions are given in Table I.

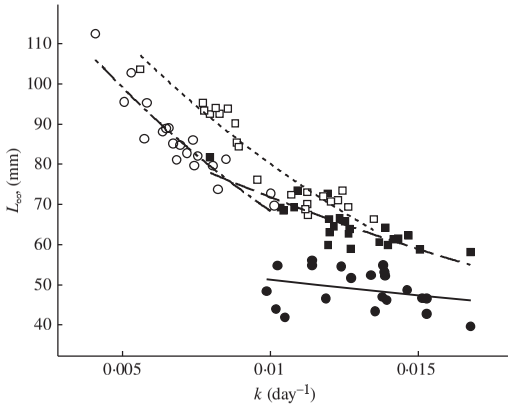


FIG. 2. The relationship between growth constant (k) and asymptotic length (L_∞) parameters estimated from the four populations (individuals plotted) of *Pungitius pungitius*: Baltic Sea (● [——]), White Sea (■, [– –]), Bynästjärnen (○, [– – –]) and Pyöreälampi (□, [·····]). The curves are exponential models (equation 2) fitted for each population. The parameters of the fitted curves are given in Table I.

and the high accuracy of measuring L_S indicate that the artefact is unlikely to affect the present analysis (Appendix SII, Supporting Information).

A previous study on the same data provides a detailed analysis of the effects of sex and family structure on fish growth (Herczeg *et al.*, 2012). In this article, the focus is on the effect of growth parameters on the predicted optimal growth strategy at different levels of predator-induced mortality. The statistics are therefore restricted to the estimation of mean and 95% C.I. of the growth parameters in the four populations separately (Table I).

OPTIMAL GROWTH STRATEGY

It was assumed that fish experience a constant level of growth-independent background mortality. The probability of surviving the first year as a juvenile was set at 40%. Fish >1 year (adults) had 80% probability of survival to a year. The values were chosen to give the modelled fish an expected life span of *c.* 3·8 years, which corresponds to the observed 3–5 years average life span of the *P. pungitius* that have reached the adult stage (Heins *et al.*, 2003). The highest known age is 7 years (Herczeg *et al.*, 2009*a*). The annual survival values translate to daily survival probabilities $s_{b,juvenile} = [(1-0.4)365^{-1}]100\% \approx 99{\cdot}75\%$ and $s_{b,adult} = [(1-0.8)356^{-1}]100\% \approx 99{\cdot}94\%$ for juveniles and adults, respectively.

In addition to background mortality, the increased mortality that results from the exposure and vulnerability of fish to predators while foraging was modelled based on the following assumptions: (1) foraging-induced mortality rate m_f is constant for each hour h spent foraging, (2) the volume of a fish (V) equals the cube of its length (L) multiplied by an allometric constant a (*i.e.* $V = aL^3$), (3) the daily increase in fish volume equals the amount of resources R foraged, scaled by the resource conversion efficiency c [*i.e.* $V(t) - V(t-1) = cR$] and (4) the volume of foraged resources is the product of foraging rate φ, fish volume V and the number of hours h spent in foraging each day ($R = \varphi hV$).

Assumption (1) makes daily survival probability a negative exponential function of foraging time: $s_g t = e^{-mht}$, where $h = R(\varphi V)^{-1}$, $R = [V(t) - V(t-1)]c^{-1}$ and $V = aL^3$ based on assumptions (2)–(4) described above. Parameter a cancels out and daily survival probability s_g becomes an exponentially decreasing function of the relative change in the cube of L between subsequent time steps:

$$s_g(t) = e^{-\left[m_f(c\varphi)^{-1}\right]\left\{\left[L^3(t)-L^3(t-1)\right]\left[L^3(t-1)\right]^{-1}\right\}}. \quad (3)$$

Variation in the foraging mortality was concentrated on and it was assumed that resource conversion efficiency c and foraging rate φ are constant. The model was therefore simplified by defining a single parameter for predator-induced mortality $m_p = m_f(c\varphi)^{-1}$ that scales the effect of fish growth to the additional mortality rate it causes. As the other parameters are fixed, all variations in predator-induced mortality m_p are attributable to foraging ($m_p \sim m_f$). Fish mortality is thus dependent on the relative growth rate of fish volume, which is a function of foraging activity (*i.e.* potential exposure to predation). It was assumed that foraging increases the exposure of fish to predators, which makes foraging-related mortality dependent on fish growth (*i.e.* size change) and not fish size *per se*. Hence, in this model, simply being larger does not equate to increased foraging effort.

Pungitius pungitius that mature early remain smaller than those maturing later (Shimada *et al.*, 2011). Two scenarios were analysed for the age of fish maturity: (1) all fish matured at the age of 1 year and (2) fish from the sea populations matured at the age of 1 year and pond populations at the age of 2 years (Herczeg *et al.*, 2009*a*; Shimada *et al.*, 2011). Thus, the fish had to exceed both a minimum size threshold and an age threshold to be able to reproduce. *Pungitius pungitius* in the wild can produce several clutches during the reproductive season (late spring to early summer), but the model here was simplified by pooling annual reproduction effort into a single reproductive event per year. The dates of reproductive events were thus $t_{rep} = 365c$, where $c = 1, 2, \ldots, \tau$ for the fish that mature at the first year and $c = 2, 3, \ldots, \tau$ for the fish that mature at the second year, where the maximum fish age τ was 10 years.

Fecundity [$f(t_{rep})$] was modelled as a linear function of L_S, based on the observed size dependency of egg production (Herczeg *et al.*, 2010*a*). The number of hatching eggs that results from each reproductive event is calculated according to the equation:

$$f(t_{rep}) = \begin{cases} 0, & L(t_{rep}) < L_{thr} \\ s_h[\alpha L(t_{rep}) + \beta] & L(t_{rep}) \geq L_{thr}. \end{cases} \quad (4)$$

The coefficients of the linear size dependency of fecundity were estimated as $\alpha = 2{\cdot}96$ and $\beta = -66{\cdot}3$ (Herczeg *et al.*, 2010*a*). These parameters determine the threshold size of *c.* 26 mm where an individual is only large enough to produce one offspring: $L_{thr} = (1 - s_h\beta)(s_h\alpha)^{-1}$. A larger data set would probably show a non-linear relation between individual size and fecundity, but the present simulations with different fecundity functions suggest that non-linearity will have only a modest, quantitative effect on the results. It was assumed that 10% of eggs survive and hatch ($s_h = 0{\cdot}1$). This value scales fitness, but does not otherwise affect the results. Fitness was calculated as the life-time reproductive success (R_0), which is the product of fecundity at each day t and the probability of surviving from day 1 to day t, summed over the maximum fish life span measured in days, $T = 365\,\tau$:

$$R_0 = \sum_{t=1}^{T} [f(t) \prod_{i=1}^{t} s_g(i) s_b(i)]. \quad (5)$$

The optimal growth strategy was L_∞ and the associated k on the trade-off function (equation 2; Fig. 1) that maximized the fitness (R_0) of an individual. The optimum L_∞ was determined using a numerical search out of 1000 values in the range $k = k_{min}, \ldots, k_{max}$, which was determined separately for each population (Appendix SIII, Supporting Information).

© 2012 The Authors
Journal of Fish Biology © 2012 The Fisheries Society of the British Isles, *Journal of Fish Biology* 2013, **82**, 318–331

The optimal growth strategy was calculated over a range of predator-induced mortality rates (m_p) to see how the fish growth changes with predator-induced mortality and to allow comparison of the four populations at different mortality rates. The value of the predator-induced mortality that gives individuals a unit fitness ($R_0 = 1$), which indicates the highest viable rate of predation-induced mortality, was also calculated. In addition, the life span associated with the optimal growth strategy at different levels of predator-induced mortality was analysed. The expected life span was calculated as a weighted average, where fish age ($t = 1 \ldots T$) is weighted by the probability of surviving to that age. The probability of surviving to a given age was calculated as the product of daily background survival and predator-induced survival (equation 3). As estimates of the background mortality in natural populations were not available, a more detailed assessment of the interaction between the two sources of mortality was not attempted.

RESULTS

THE VON BERTALANFFY MODEL PARAMETERS

The growth constant of the pond populations (Bynästjärnen and Pyöreälampi) was *c*. 33% lower than the intrinsic growth rate of the sea populations (Table I and Figs 1 and 2). The L_∞ at the pond populations was on average 26 mm (46%) higher than in the sea populations (Table I and Figs 1 and 2). The intercept (L_{int}) of the trade-off function, which indicates the theoretical maximum fish length, was on average 149 mm. This is 66 mm (80%) higher than population average for L_∞ and 24 mm (21%) higher than the largest known specimen of *P. pungitius* (115 mm; Merilä, 2006). The maximum fish size for the sea populations (on average 83 mm; Fig. 1) was 46% larger than the observed average in these populations (Table I). The differences between populations also depend on other factors (such as sex and family), which have been analysed elsewhere (Herczeg *et al.*, 2012). The statistical evaluation of the parameter differences between populations was therefore restricted to the overlap of 95% C.I. (Table I).

There was a clear negative correlation between k and L_∞ across individuals in each population (Fig. 2), except in the Baltic Sea where the steepness of the trade-off function was not statistically different from zero (Table I). The exponential trade-off function explained 72–91% of the variation in L_∞, except for the Baltic Sea, where only 9% of the variation was explained. The pond populations had a wider range of individual k and L_∞ than the sea populations (Fig. 2). The trade-off function was steeper in pond than sea populations.

MODEL PREDICTIONS

The optimal L_∞ was high and remained unchanged over a range of low values of predator-induced mortality (Fig. 3). At low predator-induced mortality, the optimal L_∞ was higher for the two ponds than for the two sea populations. The optimal L_∞ decreased with increasing predator-induced mortality rates and the populations converged to the same optimum length. The mortality level where the optimal size started to decrease was highest in sea populations, especially the Baltic Sea, where the trade-off between intrinsic growth rate and L_∞ was less steep than in other populations. A delayed maturation from 1 to 2 years in the pond populations had a

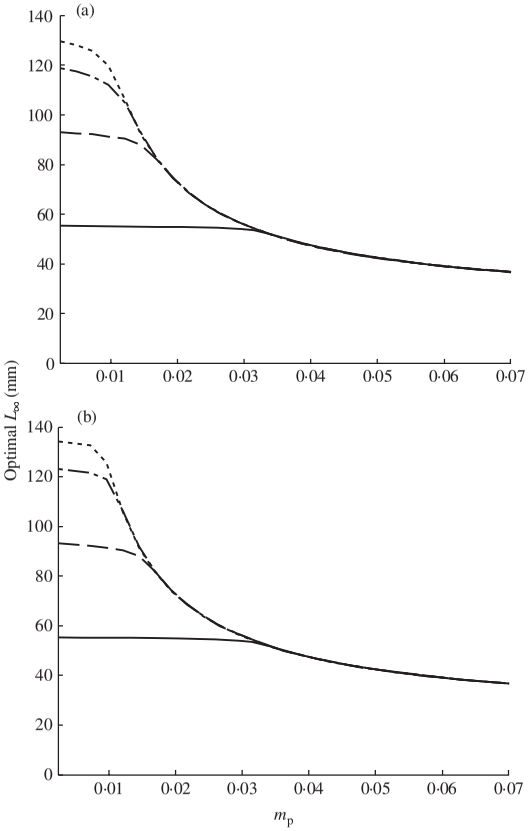


FIG. 3. Optimal asymptotic length (L_∞) in relation to predator-induced mortality rate (m_p) in *Pungitius pungitius* of the four populations: Baltic Sea (——), White Sea (– –), Bynästjärnen (– · – ·) and Pyöreälampi (– – –). The age of maturity in the pond populations (Bynästjärnen and Pyöreälampi) is (a) 1 or (b) 2 years. The age of maturity in the sea populations is 1 year in both cases.

small, quantitative effect on the optimal L_∞ that was visible only at low values of predator-induced mortality [Fig. 3(b)].

Fitness (R_0), measured as the expected life-time offspring production, decreased with predator-induced mortality (Fig. 4). When all fish matured at the age of 1 year [Fig. 4(a)], the pond populations had a higher R_0 than the sea populations under low rates of predator-induced mortality. Sea populations had a slightly higher R_0 than pond populations at high mortalities [Fig. 4(a)]. When the maturation of fish in pond populations was delayed from 1 to 2 years, the R_0 of the pond populations decreased and the pond populations had a noticeably lower R_0 than the sea populations at high

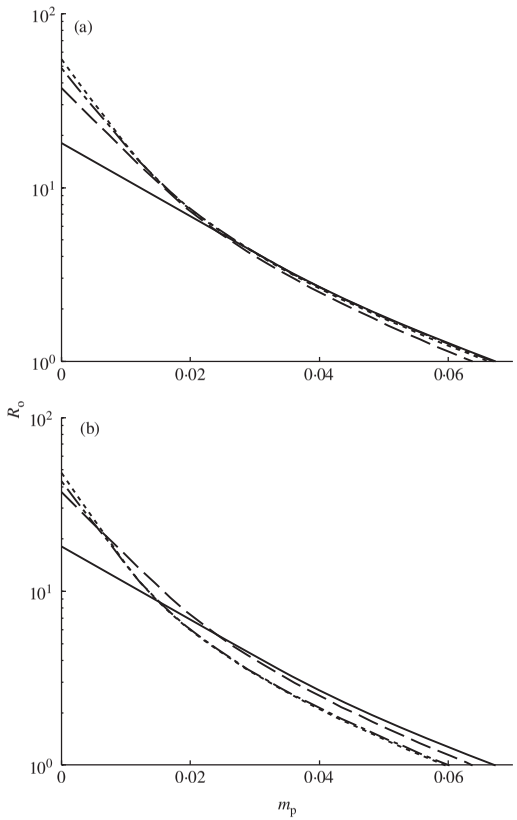


FIG. 4. Fitness (R_0) of the optimum-sized *Pungitius pungitius* in relation to predator-induced mortality rate (m_p) in the four populations: Baltic Sea (——), White Sea (– –), Bynästjärnen (– · –) and Pyöreälampi (– – –). The age of maturity in the pond populations (Bynästjärnen and Pyöreälampi) is (a) 1 or (b) 2 years. The age of maturity in the sea populations is 1 year in both cases. Note the logarithmic *y*-axis scale.

mortality rates [Fig. 4(b)]. An increase in background mortality led to a decrease in the highest viable rate of predator-induced mortality (m_p). When the pond populations matured at the age of 2 years, their tolerance of m_p was lower than that of the sea populations (Table I).

The expected life span was 3·8–3·9 years when fish experienced only background mortality (*i.e.* $m_p = 0$) and life span decreased with increasing level of m_p (Fig. 5). Fish from the sea populations had a higher expected life span than those from the pond populations for any given level of m_p. Life span decreased with decreasing optimal fish size (Fig. 3), resulting in a positive relationship between size and life span. The age at maturity (1 or 2 years) does not affect the expected life span because

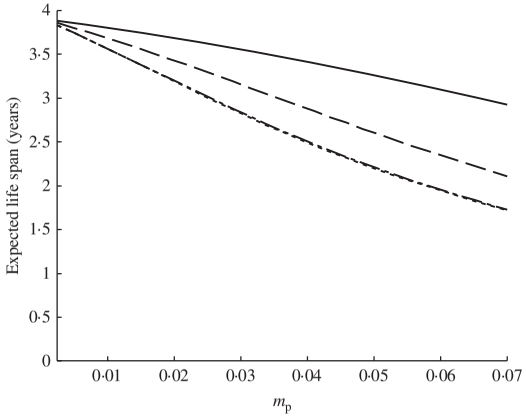


FIG. 5. Expected life span of optimal-sized *Pungitius pungitius* in relation to predator-induced mortality rate (m_p) in the four populations: Baltic Sea (——), White Sea (– –) , Bynästjärnen (– · –) and Pyöreälampi (– – –). The life span is calculated from the hatching of eggs to individual death.

the magnitude and duration of juvenile mortality were assumed to be the same in all populations.

DISCUSSION

The present analyses demonstrated that (1) a negative relationship between growth constant and asymptotic size, (2) a positive correlation between growth constant and predation risk and (3) variation in m_p can result in markedly different optimal growth strategies and lead to gigantism when predation risk is negligible. When m_p is high, fitness is more limited by the ability of individuals to mature and grow sufficiently large for reproduction than by the maximum number of offspring allowed by their body size. Individuals facing high risk of m_p should therefore mature early and grow fast to reproduce at least once, although their small body size will entail reduced fecundity. In contrast, when mortality due to predation is low, individuals can capitalize on the high fecundity enabled by a large body size and the higher probability of surviving through several reproductive events. These theoretical results fit perfectly to the observed gigantism in isolated pond populations of *P. pungitius* (Kuusela, 2006; Herczeg *et al.*, 2009*a*) and the divergent growth strategies between marine and pond populations (Herczeg *et al.*, 2012). Similar effects of varying predation pressure have been found for instance in guppy *Poecilia reticulata* Peters 1859 (Reznick & Endler, 1982; Reznick *et al.*, 1990).

Fennoscandian *P. pungitius* have gone through adaptive divergence in numerous traits after recolonizing the area following the last glaciations (Herczeg *et al.*, 2009*a*, 2012). Marine fish seem to be predation-adapted with their small body size, high growth constant, early maturation, low fecundity, fully developed body armour, lack

of developmental constraints from group living and by being shy and non-aggressive with low feeding activity (Gonda *et al.*, 2009; Herczeg *et al.*, 2009*a*, *b*, *c*, 2010*a*, *b*, 2012; Herczeg & Välimäki, 2011). Pond fish in turn seem to be competition-adapted with their large body size, low growth constant, possible later maturation, higher fecundity, reduced or lost body armour, strong developmental deficit from group living and by being bold and aggressive with high feeding activity. The main selective force behind these population differences has been assumed to be predation (and intraspecific competition in the absence of predation; Herczeg *et al.*, 2009*a*). This assumption has, however, never been tested directly. Here, the idea that the extreme difference between marine and pond populations in the presence or absence of size-unlimited sympatric predatory fish could be responsible for the divergence in size and growth strategy (Herczeg *et al.*, 2009*a*; Herczeg & Välimäki, 2011) is probed, and a strong support has been found for it. Further, the present modelling approach revealed a number of interesting points about how survival and fecundity shape the optimal growth strategy under different size-unlimited predation caused mortalities.

Early maturation has a very strong effect on R_0 that early theoretical studies suggested that it should hardly ever be beneficial to postpone reproduction beyond the first opportunity (Cole, 1954). Later analyses have shown that either fecundity or survival must increase markedly before delayed reproduction becomes a viable life-history strategy (Charnov & Schaffer, 1973; Bulmer, 1994). Delaying maturity from 1 to 2 years, as seemingly is the case in pond populations of *P. pungitius* (Herczeg *et al.*, 2009*a*; Shimada *et al.*, 2011), should therefore be beneficial only if the R_0 costs are balanced by a marked increase in the expected number of offspring produced in later reproductive events. Different mechanisms may be involved to give later maturity an advantage. First, early maturity itself is costly as it decreases the asymptotic body size compared to later maturing individuals (Charnov, 1993; Berrigan & Charnov, 1994). Second, larger body size may enhance competitive ability in direct confrontation between individuals (Roff, 1992) and lead to higher fecundity (Heins *et al.*, 2003, 2005; Herczeg *et al.*, 2010*a*). It is also possible that the survival costs of reproduction play a substantial role (Bell, 1980; Kuparinen *et al.*, 2012), although this factor was not included in the model.

The benefits from early maturation diminish if an individual can expect to survive to a later reproductive event and be able to produce more offspring. The *P. pungitius* in some ponds have been found to live almost twice as long as their marine conspecifics (Herczeg *et al.*, 2009*a*). The model indicates, however, that for a given level of m_p, the fish of the sea populations should live longer than the fish of the pond populations (Fig. 5). The shorter life span of pond fish is a consequence of their growth strategy, which makes them more sensitive to the effects of predation. The fish of the pond populations must therefore experience substantially lower m_p than the fish in sea populations. If background survival is high and a fish lives in a low predation environment, such as isolated ponds, the costs of delayed reproduction may be sufficiently low to be overtaken by higher fecundity allowed by a larger body size that results from late maturity and low m_p.

This study required that fish of different populations are reared in the same conditions, so that the genetic differences in growth parameters are not masked by different growth conditions. This was easiest to achieve using common-garden data, with a drawback that rearing conditions were likely to differ from those experienced by

fish in their natural environment. This is a problem common to all common-garden studies, and can only be avoided by use of reciprocal transplant experiments, which are logistically hard to conduct. The asymptotic size estimates of the laboratory-reared fish (Table I) are, however, close to the sizes of the adult fish caught in wild from the same populations (Herczeg *et al.*, 2009*a*). Hence, it seems unlikely that the results are strongly biased by use of the common-garden data. Any such bias would be minor compared to the benefits of standardized conditions allowed by the common-garden environment.

In conclusion, it was found that optimal growth strategy under a trade-off between k and L_∞ favours slow k and high L_∞ in the relatively predator-free pond populations, where the fish can grow to unusually large size. A faster k is optimal in high predation risk sea environments, although it comes with the cost of fish having a smaller L_∞. The present theoretical model of fish growth and life-time reproduction thus seems to be a sufficient explanation of gigantism and requires few assumptions, which were tested empirically using aquarium-reared fish from different populations. The model could easily be adapted for other species with data on growth-rate parameters.

We thank A. Gonda for assistance in capturing and rearing the experimental fish. The Oulanka Research Station provided facilities for part of the field work. We received financial support from the Academy of Finland (grant number 138309 to S.A. from A. M. Markkola, number 128716 to G.H.; number 132828 to A.K. and numbers 129662, 134728 and 218343 to J.M.) and the University of Oulu research fellowship (S.A.).

SUPPORTING INFORMATION

Supporting Information may be found in the online version of this paper:

APPENDIX SI. The relationship between growth constant and growth rate.

APPENDIX SII. Is the trade-off between k and L_∞ an artefact?

APPENDIX SIII. The feasible range of growth constant (k) values.

References

Abrams, P. A. & Rowe, L. (1996). The effects of predation on the age and size of maturity of prey. *Evolution* **50,** 1052–1061.

Andersson, M. (1994). *Sexual Selection.* Princeton, NJ: Princeton University Press.

Arendt, J. D. (2010). Size-fecundity relationships, growth trajectories, and the temperature-size rule for ectotherms. *Evolution* **65,** 43–51.

Atchley, W. R. (1984). Ontogeny, timing of development, and genetic variance-covariance structure. *American Naturalist* **123,** 519–540.

Bănărescu, P. M. & Paepke, H.-J. (2001). *The Freshwater Fishes of Europe*, Vol. 5/III. Wiebelsheim: AULA-Verlag.

Bell, G. (1980). The costs of reproduction and their consequences. *American Naturalist* **116,** 45–76.

Berrigan, D. & Charnov, E. L. (1994). Reaction norms for age and size at maturity in response to temperature: a puzzle for life historians. *Oikos* **70,** 474–478.

von Bertalanffy, L. (1938). A quantitative theory of organic growth (inquires on growth laws II). *Human Biology* **10,** 181–213.

Biro, P. A., Abrahams, M. V., Post, J. R. & Parkinson, E. A. (2004). Predators select against high growth rates and risk-taking behaviour in domestic trout populations. *Proceedings of the Royal Society B* **271,** 2233–2237.
Biro, P. A., Abrahams, M. V., Post, J. R. & Parkinson, E. A. (2006). Behavioural trade-offs between growth and mortality explain evolution of submaximal growth rates. *Journal of Animal Ecology* **75,** 1165–1171.
Blanckenhorn, W. U. (2000). The evolution of body size: what keeps organisms small? *Quarterly Review of Biology* **75,** 385–407.
Blanckenhorn, W. U. & Demont, M. (2004). Bergmann and converse Bergmann latitudinal clines in arthropods: two ends of a continuum? *Integrative and Comparative Biology* **44,** 413–424.
Bulmer, M. (1994). *Theoretical Evolutionary Ecology.* Sunderland, MA: Sinauer Associates.
Calow, P. (1982). Homeostasis and fitness. *American Naturalist* **120,** 416–419.
Charnov, E. L. (1993). *Life-History Invariants.* Oxford: Oxford University Press.
Charnov, E. L. & Schaffer, W. M. (1973). Life-history consequences of natural selection: Cole's results revisited. *American Naturalist* **107,** 791–792.
Cole, L. C. (1954). The population consequences of life history phenomena. *Quarterly Review of Biology* **29,** 103–137.
Day, T. & Rowe, L. (2002). Developmental thresholds and the evolution of reaction norms for age and size at maturity. *American Naturalist* **159,** 338–350.
Dmitriew, C. M. (2010). The evolution of growth trajectories: what limits growth rate? *Biological Reviews* **86,** 97–116.
Gonda, A., Herczeg, G. & Merilä, J. (2009). Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain. *Proceedings of the Royal Society B* **276,** 2085–2092.
Heins, D. C., Johnson, J. M. & Baker, J. A. (2003). Reproductive ecology of the nine-spined stickleback from south-central Alaska. *Journal of Fish Biology* **63,** 1131–1143.
Heins, D. C., Baker, J. A., De Silva, G. & Birden, E. L. (2005). Clutch characteristics of two populations of nine-spined stickleback from south-central Alaska. *Journal of Fish Biology* **67,** 873–878.
Herczeg, G. & Välimäki, K. (2011). Intraspecific variation in behaviour: effects of evolutionary history, ontogenetic experience and sex. *Journal of Evolutionary Biology* **24,** 2434–2444.
Herczeg, G., Gonda, A. & Merilä, J. (2009*a*). Evolution of gigantism in nine-spined sticklebacks. *Evolution* **63,** 3190–3200.
Herczeg, G., Gonda, A. & Merilä, J. (2009*b*). Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *Journal of Evolutionary Biology* **22,** 544–552.
Herczeg, G., Gonda, A. & Merilä, J. (2009*c*). The social cost of shoaling covaries with predation risk in nine-spined stickleback (*Pungitius pungitius*) populations. *Animal Behavior* **77,** 575–580.
Herczeg, G., Gonda, A. & Merilä, J. (2010*a*). Rensch's rule inverted – female-driven gigantism in nine-spined stickleback *Pungitius pungitius*. *Journal of Animal Ecology* **79,** 581–588.
Herczeg, G., Turtiainen, M. & Merilä, J. (2010*b*). Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. *Biological Journal of the Linnean Society* **101,** 413–416.
Herczeg, G., Gonda, A., Kuparinen, A. & Merilä, J. (2012). Contrasting growth strategies at high vs. low predation populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? *Evolutionary Ecology* **26,** 109–122.
Kuparinen, A., Hardie, D. C. & Hutchings, J. A. (2012). Evolutionary and ecological feedbacks of the survival cost of reproduction. *Evolutionary Applications* **5,** 245–255.
Kuusela, K. (2006). Isolated ninespine stickleback population, *Pungitius pungitius* (L.) in northeast Finland: large fish with reduced pelvis. *Verhandlungen des Internationalen Verein Limnologie* **29,** 1409–1412.
Leppäranta, M. & Myrberg, K. (2009). *Physical Oceanography of the Baltic Sea.* Berlin: Springer.

Lima, S. L. & Dill, L. M. (1990). Behavioural decisions made under the risk of predation–a review and prospectus. *Canadian Journal of Zoology* **68,** 619–640.
Merilä, J. (2006). Ennätyksellisen iso kymmenpiikki (*Pungitius pungitius*) Kuusamon Rytilammesta. *Luonnon Tutkija* **110,** 91–93.
Östlund-Nilsson, S., Mayer, I. & Huntingford, F. A. (2007). *Biology of the Three-Spined Stickleback.* Boca Raton, FL: CRC Press.
Peters, R. H. (1983). *The Ecological Implications of Body Size.* Cambridge: Cambridge University Press.
Ratkowsky, D. A. (1986). Statistical properties of alternative parameterizations of the von Bertalanffy growth curve. *Canadian Journal of Fisheries and Aquatic Sciences* **43,** 742–747.
Reznick, D. A. & Endler, J. A. (1982). The impact of predation of life history evolution in Trinidadian guppies (*Poecilia reticulata*). *Evolution* **36,** 160–177.
Reznick, D. A., Bryga, H. & Endler, J. A. (1990). Experimentally induced life-history evolution in a natural population. *Nature* **346,** 357–359.
Roff, D. A. (1992). *The Evolution of Life Histories.* New York, NY: Chapman & Hall.
Shimada, Y., Shikano, T., Kuparinen, A., Gonda, A., Leinonen, T. & Merilä, J. (2011). Quantitative genetics of body size and timing of maturation in two nine-spined sticklebacks (*Pungitius pungitius*) populations. *PLoS One* **6,** e28859. doi: 10.1371/journal.pone.0028859
Shine, R. (1989). Ecological causes for the evolution of sexual size dimorphism: a review of the evidence. *Quarterly Review in Biology* **64,** 419–461.
Sibly, R. & Calow, P. (1986). Why breeding earlier is always worthwhile. *Journal of Theoretical Biology* **124,** 311–319.
Stearns, S. C. (1992). *The Evolution of Life Histories.* Oxford: Oxford University Press.
Urban, M. C. (2008). Salamander evolution across a latitudinal cline in gape-limited predation risk. *Oikos* **117,** 1037–1049.
Werner, E. E. & Gilliam, J. F. (1984). The ontogenetic niche and species interactions in size structured populations. *Annual Reviews in Ecology and Systematics* **15,** 393–425.

Electronic Reference

Rohlf, F. J. (2006). *TPS Software Series.* Stony Brook, NY: Department of Ecology and Evolution, State University of New York. Available at http://life.bio.sunysb.edu/ee/rohlf/software.html.

MOLECULAR ECOLOGY

Molecular Ecology (2013) **22**, 565–582 doi: 10.1111/j.1365-294X.2012.05749.x

Molecular evolutionary and population genomic analysis of the nine-spined stickleback using a modified restriction-site-associated DNA tag approach

MATTHIEU BRUNEAUX,*[1] SUSAN E. JOHNSTON,*[1] GÁBOR HERCZEG,† JUHA MERILÄ,† CRAIG R. PRIMMER* and ANTI VASEMÄGI*‡

*Division of Genetics and Physiology, University of Turku, Vesilinnantie 5, FI-20014, Turku, Finland, †Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, PO Box 65, FI-00014, Helsinki, Finland, ‡Department of Aquaculture, Institute of Veterinary Medicine and Animal Science, Estonian University of Life Sciences, Kreutzwaldi 48, 51014, Tartu, Estonia

Abstract

In recent years, the explosion of affordable next generation sequencing technology has provided an unprecedented opportunity to conduct genome-wide studies of adaptive evolution in organisms previously lacking extensive genomic resources. Here, we characterize genome-wide patterns of variability and differentiation using pooled DNA from eight populations of the nine-spined stickleback (*Pungitius pungitius* L.) from marine, lake and pond environments. We developed a novel genome complexity reduction protocol, defined as paired-end double restriction-site-associated DNA (PE dRAD), to maximize read coverage at sequenced locations. This allowed us to identify over 114 000 short consensus sequences and 15 000 SNPs throughout the genome. A total of 6834 SNPs mapped to a single position on the related three-spined stickleback genome, allowing the detection of genomic regions affected by divergent and balancing selection, both between species and between freshwater and marine populations of the nine-spined stickleback. Gene ontology analysis revealed 15 genomic regions with elevated diversity, enriched for genes involved in functions including immunity, chemical stimulus response, lipid metabolism and signalling pathways. Comparisons of marine and freshwater populations identified nine regions with elevated differentiation related to kidney development, immunity and MAP kinase pathways. In addition, our analysis revealed that a large proportion of the identified SNPs mapping to LG XII is likely to represent alternative alleles from divergent X and Y chromosomes, rather than true autosomal markers following Mendelian segregation. Our work demonstrates how population-wide sequencing and combining inter- and intra-specific RAD analysis can uncover genome-wide patterns of differentiation and adaptations in a non-model species.

Keywords: adaptive divergence, allelotyping-by-sequencing, genome scan, next generation sequencing, single nucleotide polymorphism, stickleback

Received 15 March 2012; revision received 18 May 2012; accepted 7 June 2012

Introduction

The field of ecological genomics seeks to understand the ecological significance of genomic variation and the function of single or multiple genes in the wild (Feder & Mitchell-Olds 2003; van Straalen & Roelofs 2006; Ellegren & Sheldon 2008; Ungerer *et al.* 2008). Linking genome-wide variation with phenotype in an organism ultimately requires genome sequence information, and as a result, much of the progress in this field to date has been limited to genetic model organisms (*e.g.* Fournier-Level *et al.* 2011; Jelier *et al.* 2011). However, the recent explosion in affordable and high-throughput technologies for next generation sequencing (NGS), microarray and mass spectrometry means that genome-wide studies in non-model organisms are now much more achievable (Tautz *et al.* 2010; Ekblom & Galindo 2011), and several studies have demonstrated that novel functional genomic insights can be gained for species without fully sequenced genomes (*e.g.* Vera *et al.* 2008; Papakostas *et al.* 2012).

Correspondence: Anti Vasemägi, Fax: +358 2 333 6680;
E-mail: anti.vasemagi@utu.fi
[1]These authors contributed equally to this work.

One promising NGS approach in species lacking genome sequence information is restriction-site-associated DNA (RAD) sequencing, a method that sequences the DNA flanking specific restriction sites throughout the genome (Baird *et al.* 2008; Hohenlohe *et al.* 2010). This approach allows ultra-high coverage sequencing of the same sites over many individuals and/or populations, providing a reduced representation of the genome and sufficient read depth for the detection of sequence polymorphisms, particularly single nucleotide polymorphisms (SNPs), whilst using a relatively small number of sequencing runs. The RAD method has been successfully applied in a number of non-model organisms to develop thousands of SNPs (*e.g.* Scaglione *et al.* 2012), study hybrid zones (Hohenlohe *et al.* 2011) and to create high-density genetic linkage maps (Baxter *et al.* 2011; Miller *et al.* 2012). The aforementioned studies have generally adopted a genotyping-by-sequencing approach, where sequencing and SNP genotyping are conducted on an individual basis. However, several empirical and theoretical studies have evaluated the alternative strategy of allelotyping-by-sequencing, which estimates allele frequencies from DNA pools rather than from individual genotypes (van Tassell *et al.* 2008; Futschik & Schlötterer 2010; Mullen *et al.* 2012). This approach enables cost-effective SNP discovery, as well as fast and accurate allele frequency estimation from DNA pools for genome-wide population genetic studies (Futschik & Schlötterer 2010). Therefore, allelotyping-by-sequencing is expected to be a very cost-effective approach for population genetic studies of multiple populations at a genome-wide scale.

The nine-spined stickleback (*Pungitius pungitius* L.) is a small fish distributed across marine and freshwater habitats throughout the Northern Hemisphere. In Fennoscandia (Fig. 1), nine-spined stickleback populations share a common ancestry after recolonization of the region from the southern latitudes following the last glacial maximum (Shikano *et al.* 2010c; Teacher *et al.* 2011). There is extensive habitat variation throughout this region, through both abiotic factors, such as salinity, temperature and habitat structure, and biotic factors, such as prey abundance, competition and predation. As a result, differences in selection pressures are expected to lead to adaptive divergence between populations. Earlier works have revealed significant differences in morphological, behavioural and life-history traits between freshwater and marine nine-spined stickleback populations, with evidence supporting the idea that adaptation in freshwater pond populations is driven by intra-specific competition, whereas adaptation in marine populations is driven by predation. Freshwater populations typically exhibit higher aggression and boldness (Herczeg *et al.* 2009a; Herczeg & Välimäki 2011), reduced or absent body armour (Herczeg *et al.* 2010), gigantism and variation in growth strategies (Herczeg *et al.* 2009b, 2012; Shimada *et al.* 2011) and divergent brain architecture (Gonda *et al.* 2009, 2012) in comparison with marine populations.

To date, population genetic and linkage mapping studies in nine-spined stickleback have relied on markers developed from species-specific genomic libraries (Shapiro *et al.* 2009), or more recently, sequence information from the related three-spined stickleback, *Gasterosteus aculeatus* (Shikano *et al.* 2010a,b). Nine-spined and three-spined sticklebacks shared a common ancestor more than 13 million years ago (Bell *et al.* 2009) and have 21 visible chromosomes (Chen & Reisman 1970). Linkage mapping of North American nine-spined sticklebacks showed conserved synteny of microsatellite marker locations between the two genera (Shapiro *et al.* 2009); linkage groups (LGs) in the nine-spined stickleback can be referred to in terms of their three-spined counterparts. Despite this synteny, there is evidence that convergent phenotypic evolution between these genera has a divergent genetic architecture. For example, Shapiro *et al.* (2009) uncovered major quantitative trait loci (QTL) associated with morphological traits such as pelvic reduction and lateral plate number variation, but these did not correspond to QTL for similar traits within the three-

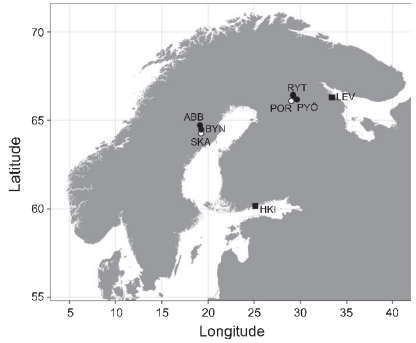


Fig. 1 Sampling locations of nine-spined stickleback in Fennoscandia. Marine, pond and lake populations are marked as filled squares, filled circles and open circles, respectively. For population abbreviations, see Table 1.

spined stickleback (Cresko *et al.* 2004; Shapiro *et al.* 2004; Coyle *et al.* 2007). Similarly, the sex-determining locus in nine-spined sticklebacks has been mapped to chromosome 12 (LG XII) in both North America and Eastern European lineages (Shapiro *et al.* 2009; Shikano *et al.* 2011), whereas that of the three-spined stickleback has been mapped to chromosome 19 (LG XIX; Peichel *et al.* 2004). Recently, several high-density genome scans using RAD sequencing have provided new and valuable information on genomic regions affected by adaptation in three-spined stickleback (Hohenlohe *et al.* 2010; Deagle *et al.* 2011; Roesti *et al.* 2012), and full genome sequencing of multiple marine and freshwater three-spined stickleback individuals has also been carried out (Jones *et al.* 2012). However, the nine-spined stickleback still lacks a comprehensive population genomic perspective on the genome-wide patterns of variation and differentiation. Such studies on the nine-spined stickleback represent a promising opportunity to generalize findings from the three-spined stickleback and indicate whether the same or different chromosomal regions and genes are involved in adaptation to similar environments in different species.

In this study, we combined information from next generation sequencing in the nine-spined stickleback with genomic resources from the related three-spined stickleback. This allowed us to characterize, for the first time, a genetic profile for a significant proportion of the nine-spined stickleback genome. By developing a novel genome complexity reduction protocol (referred to as paired-end double RAD, or PE dRAD) on pooled DNA samples from a number of populations, we demonstrate how inter- and intra-specific RAD analysis can reveal novel genome-wide patterns of differentiation between populations of a non-model species.

Materials and methods

Population sampling

Fin clips were collected from two marine, two lake (surface area > 20 ha) and four pond-dwelling (surface area < 5.8 ha) populations of nine-spined stickleback across Fennoscandia between 2006 and 2007 (Fig. 1; Table 1; see Shikano *et al.* 2010c for further details). Of these, one marine, one lake and two pond populations were selected from Baltic Sea and White Sea drainage systems (Table 1). DNA was extracted using a proteinase K digestion protocol and a silica-fine based purification method (Aljanabi & Martinez 1997; Elphinstone *et al.* 2003). DNA quality was checked in 1% agarose gel, and DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer. For each population, equimolar amounts (10 ng/μL) of high-quality genomic DNA from 48 individuals were pooled and concentrated to 30 ng/μL using a vacuum concentrator (5301, Eppendorf).

RAD tag library creation and sequencing

Restriction-site-associated DNA libraries were generated using the protocol outlined in Baird *et al.* (2008), with two key modifications to improve efficacy of allelotyping-by-sequencing: (i) the 5′ end of the Modified Adapter 1 was labelled with biotin using streptavidin-coupled Dynabeads® (MyOne™ C1, Invitrogen GmbH), and a magnet was used to efficiently separate desired fragments prior to PCR; (ii) instead of physically shearing the DNA, we used a second restriction enzyme (*Hae*III) to create small fragments suitable for sequencing. These adaptations simplified the library preparation protocol and produced fully overlapping reverse reads, maximizing the reverse read coverage. As this new method combines the use of double restriction and paired-end sequencing, we refer to it as a paired-end double-RAD (PE dRAD) approach.

For each pooled sample, 1200 ng of genomic DNA was digested for 20 min at 37 °C in a 50 μL reaction containing 30 U of *Eco*RI in 1 × NEBuffer 4 (New England Biolabs, hereafter denoted as 'NEB'), before being heat-inactivated for 20 min at 65 °C. 1 μL of 2 μM Modified Adapter 1 (top: 5′-Biotin-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′, bottom: 5′-Phos-AATTAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG-3′) was added to 45 μL of the restriction product, along with 1 μL of 100 mM rATP (Promega), 0.5 μL 10 × NEBuffer 4 and 2.5 μL (1000 U) T4 DNA Ligase (400 U/μL, NEB). A ligation reaction was carried out at room temperature for 30 min, before heat-inactivation for 20 min at 65 °C. The ligation product was then cut by adding 2 μL of second restriction enzyme, *HaeIII* (20 U, NEB). These samples were digested for 20 min at 37 °C, heat-inactivated for 20 min at 75 °C, purified using NucleoSpin Extract II columns (Macherey-Nagel) and eluted in 16 μL of 10 mM Tris-HCl pH 8.5. The sample was then mixed with 16 μL of 2 × Washing and Binding buffer (Invitrogen GmbH). DNA fragments containing Modified Adapter 1 were immobilized using streptavidin-coupled Dynabeads® (MyOne™ C1, Invitrogen GmbH), washed three times and eluted in 34 μL of water. 6.5 U of Klenow exo−(NEB) was used to add adenine (10 mM, dATP) overhangs on the 3′ end of the DNA fragments at 37 °C, followed by heat-inactivation for 30 min at 65 °C. The sample was then immobilized, washed twice and eluted in 14 μL of water. After the purification, 1 μL of 2 μM Modified Adapter 2 (top: 5′-Phos-XXXAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG-3′, bottom: 5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTXXXT-3′,

Table 1 Information and basic summary statistics for each population subject to PE dRAD sequencing

Population	Location	Coordinates	Habitat	Drainage	Lane and barcode	No. forward reads	No. reverse reads	Mean SNP alignment depth
ABB	Abbortjärn, Sweden	64°29′N, 19°26′E	Pond	Baltic Sea	3_TTG	396 861	428 807	6.36
BYN	Bynastjärnen, Sweden	64°27′N, 19°27′E	Pond	Baltic Sea	1_CAC	577 420	599 202	10.06
PYÖ	Pyöreälampi, Finland	66°16′N, 29°26′E	Pond	White Sea	3_CTT	652 455	675 123	9.13
RYT	Rytilampi, Finland	66°23′N, 29°19′E	Pond	White Sea	1_CTT	175 685	187 008	3.06
POR	Iso-Porontima, Finland	66°13′N, 29°16′E	Lake	White Sea	6_TTG	321 539	333 744	4.56
SKA	Västre-Skavträsket, Sweden	64°26′N, 19°27′E	Lake	Baltic Sea	5_CAC	78 983	81 633	1.24
HKI	Helsinki, Finland	60°12′N, 25°11′E	Marine	Baltic Sea	2_TCT	446 765	485 602	7.14
LEV	Levin Navolok Bay, Russia	66°18′N, 33°24′E	Marine	White Sea	5_TCT	361 187	376 011	4.79

where XXX is a barcode sequence specific to the population pool, Table 1) was ligated to the DNA fragments with adenine overhang at room temperature, as in the first ligation reaction with Modified Adapter 1. The sample was immobilized, washed and eluted in 50 μL of water, and 10 μL of this product was used for PCR amplification in a 50 μL reaction volume containing 0.5-μL Platinum Pfx DNA polymerase (Invitrogen), 20 mM dATPs, 100 mM $MgSO_4$, 50 μM of primers (forward: 5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTA-3′; reverse: 5′-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-3′) and 5 μL of 10 × amplification buffer (Invitrogen). The PCR program consisted of an initial denaturation at 94 °C for 2 min, followed by 18 cycles of denaturation in 94 °C for 15 s, annealing at 60 °C for 30 s, extension at 68 °C for 30 s, with a final extension at 72 °C for 5 min. PCR products were purified using a MinElute PCR Purification Kit (Qiagen) and eluted in 12 μL of 10 mM Tris-HCl pH 8.5. The purified PCR products were then separated on a 2% agarose gel, and the bands within the size range 250–400 bp were excised with sterile scalpels. DNA within the excised band was purified using MinElute Gel Extraction Kit (Qiagen), eluted in 27 μL of 10 mM Tris-HCl pH 8.5 and diluted to 10 nM. Paired-end sequencing was carried out on the Illumina GAII sequencing platform, indexed by population (Table 1).

De novo assembly of consensus sequences

Forward and reverse sequence reads were assigned to each population based on barcode information (Table 1), and restriction site and barcode sequences were removed. *De novo* assembly of consensus sequences was conducted incorporating read quality information in the software CAP3 (Huang & Madan 1999) for forward and reverse reads separately, and consensus sequences matching the median-trimmed read lengths (69 and 68 bases for forward and reverse reads, respectively) were retained. All consensus sequences were aligned to one another using *blastn* in BLAST+ v2.2.25 (Altschul *et al.* 1990) to identify repetitive sequence motifs and identical or extremely similar consensus sequences (sometimes observed in CAP3 assemblies; Shahin *et al.* 2012). Consensus sequences were discarded if they contained motifs of ≥ 15 bases which had ≥ 50 matches in the BLAST+ alignment (where thresholds were empirically selected based on the distribution of motif lengths and match count), and a common consensus sequence was selected where two consensus sequences had a unique match with ≥ 62 matching bases and no gaps. Finally, consensus sequences were screened for microsatellite repeats (mono-, di- or tri-nucleotide of length ≥ 10 bases, respectively) using SCIROKO v3.4 (Kofler *et al.* 2007).

Read alignment and SNP discovery

The overall miscall rate on the Illumina platform is around 1% (Nielsen *et al.* 2011), and the average quality score of each base decreases towards the 3′ end of the sequence read. To minimize false SNP detection, bases were removed from the 3′ end of all reads until the Phred quality score was ≥20 (equivalent to a 1% error rate) using Fastq Quality Trimmer implemented in Galaxy Tools (Goecks *et al.* 2010); remaining reads with a length of <40 bases were discarded. Read alignment to the consensus sequences and SNP discovery was carried out using the *map* function in MAQ v0.7.1 (Li *et al.* 2008). The positions of putative SNP loci on the consensus sequences were estimated using the *assemble* and *cns2snp* functions, utilizing information from each population individually and all populations combined, to identify putative SNPs within and between populations, respectively. Alignment information for each putative SNP was extracted from individual populations using the *pileup* function.

dc_892_14

SNP determination and allele frequency estimation

Read alignment information at each putative SNP locus was concatenated and further validation conducted in R v2.14.1. Alleles with a frequency below the estimated error rate (i.e. ≤ 0.01) were removed to reduce the likelihood of false allele detection. To avoid false SNP detection because of repetitive regions and/or sequencing error, the remaining SNP loci were retained if they met the following selection criteria: biallelic; read alignment depth (coverage) of ≥ 10 and ≤ 1000; ≥ 3 reads per SNP allele; no more than one additional SNP on the same consensus sequence; and not falling $\leq$ 5 bases of identified microsatellite regions. Uniquely to our study, a power failure experienced during the sequencing affected two positions at the 3′ end of the reverse reads; any SNPs detected at these positions were removed. In cases where more than one SNP was detected on the same consensus sequence, only those with two haplotypes were retained, with the SNP closest to the 5′ end of the sequence scored. Finally, the read alignment depths and allele frequencies at each SNP locus were calculated across all populations and within each population/habitat.

Genetic diversity and genetic relationships between populations

An unbiased estimator of gene diversity, H_s (Nei & Roychoudhury 1974), and proportion of polymorphic loci were calculated for all and for a subset of SNPs (2067 SNPs with >60 overall coverage; at least one read observed per population). SNP variability estimates were compared to published microsatellite diversity values for the same populations (12 loci; H_E, H_O, PIC and proportion of polymorphic loci; Shikano *et al.* 2010c). Cavalli-Sforza chord distance (Cavalli-Sforza & Edwards 1967) and a neighbour-joining tree building algorithm were used to infer the genetic relationships between populations. Several trees were constructed based on different number of SNPs (3624 markers with at least one read observed per population and 589 markers with at least four reads per population). Branch support was evaluated by performing 1000 bootstrap replications; all analyses within this section were conducted using PHYLIP v3.69 (Felsenstein 2004).

Mapping of the consensus sequences to a closely related reference genome

To perform a genome scan for the nine-spined stickleback, the three-spined stickleback (*Gasterosteus aculeatus*) genome sequence was used as a reference to determine the approximate genomic location of the nine-spined stickleback consensus sequences. Nucleotide to nucleotide and translated nucleotide to protein alignments were carried out using *blastn* and *blastx* in BLAST+ v2.2.24, using the nucleotide top-level database and the protein all-peptides database from the Ensembl depository for three-spined stickleback as reference databases (*Ga* database release 56 for DNA and 65 for peptides, available at http://www.ensembl.org/, Flicek *et al.* 2011). Mapping locations of the consensus sequences were accepted if they met at least one of the following three criteria, tested in this order: (i) the ratio of the lowest *blastn* e-value to the second lowest e-value was $\geq 10^5$ (*blastx* results consistent with those *blastn* location were simultaneously validated); (ii) *blastx* results and *blastn* results not fulfilling criterion (i) had consistent genomic locations and an e-value product $\leq 10^{-5}$; (iii) the ratio of the two lowest *blastn* e-values was $\geq 10^3$, with the lowest e-value being $\leq 10^{-4}$, for *blastn* results not fulfilling criteria (i) or (ii); no further *blastx* results were validated after this point. These criteria were similar to those used in earlier studies with inter-specific comparisons (Stemshorn *et al.* 2005; Shapiro *et al.* 2009) and were chosen to provide a conservative sequence alignment, whilst accounting for the fact that the reference genome was not from the same species.

Estimation of consensus sequence densities along the reference genome

The quality of the sequence distributions deduced from *G. aculeatus* genome was controlled by checking the consistency of observed and expected patterns before performing the actual genome scan. The density of nine-spined consensus sequences relative to their position on the three-spined genome was estimated using a kernel density estimation implemented in R v2.14.1 (*density* function) with a Gaussian kernel and a bandwidth value (defined as the standard deviation of the kernel) of 400 kb (de Ridder *et al.* 2006). This kernel density estimation approximates the probability density function of a variable and is analogous to a continuous, smoothed histogram of a distribution. The expected distribution of RAD fragments was generated by carrying out *in-silico* digestion of the three-spined reference genome using *Eco*RI and *Hae*III, selecting fragments cut by both enzymes of 220–440 bases in length. This information was used to generate an expected density distribution to compare to the observed distribution of mapped consensus sequences. The expected distribution of exons was also generated, where exon positions were determined using the *G. aculeatus* Ensembl database. This was used for comparison with the observed distribution of consensus sequences assigned to coding sequences, that is, the consensus sequences with validated *blastx* matches. Comparisons of distributions were performed using a linear regression model (LRM) applied to the paired values of the compared distributions for the 10 752 points of the interpolation grid over the genome.

Identification of regions with elevated and reduced levels of polymorphism/variability

Density estimates were calculated for all consensus sequences and SNPs that were mapped to the reference genome. A permutation test was performed to test for non-random patterns of polymorphism and detect genomic regions exhibiting significantly elevated or decreased levels of polymorphism. Briefly, simulated distributions of polymorphic positions were generated by randomly assigning the same number of observed polymorphic SNPs to positions throughout the genome to which a consensus sequence had been mapped; new density estimates were then generated for each simulated data set and compared to the observed one. The *P*-values for high and low polymorphism levels at a given position were calculated as the proportion of simulated density estimates higher or lower than the observed density estimate at that particular position. Corresponding *q*-values were estimated using the *qvalue* package in R v2.14.1 (Storey *et al.* 2004).

Identification of significantly differentiated regions between habitats by G_{st} kernel estimation

To increase the accuracy of single locus G_{st} estimates, we grouped populations according to habitat type (marine and freshwater). G_{st} values were calculated using the formula:

$$G_{st} = H_t - H_s / H_t$$

where H_s is the average of expected heterozygosity within each habitat and H_t is the overall expected heterozygosity across all habitats. Expected heterozygosities were calculated using the formula:

$$H = 1 - a^2 b^2$$

where a and b are the allele frequencies observed within the habitat type (for H_s) or overall (for H_t). To limit the effect of sample size differences between habitats when estimating H_t, we did not calculate the overall allele frequencies from the count of each allele after pooling both habitats samples, but rather calculated the overall frequency of each allele as the average frequency within each habitat. G_{st} values were smoothed over the whole genome by using a local kernel regression within each chromosome, similar to Hohenlohe *et al.* (2010). The smoothing was performed using a kernel regression smoother implemented in R v2.14.1 (*ksmooth* function), with a normal kernel and a bandwidth value of 350 kb, defined as the standard deviation (σ) of the kernel. Two analyses were conducted using 10 and 20 as the minimum coverage per SNP, per habitat. Regions showing significantly high or low smoothed G_{st} values (*i.e.* elevated or reduced differentiation between the habitats) were identified using a permutation test within each chromosome data set, allowing the comparison of the observed kernel regression curve to a distribution of curves obtained from simulated data sets (Flori *et al.* 2009; Hohenlohe *et al.* 2010). Simulated data sets were generated by shuffling the observed G_{st} values randomly over the observed set of chromosomal positions. Corresponding *q*-values were estimated using the *qvalue* package in R v 2.14.1 (Storey *et al.* 2004).

Gene ontology enrichment test

Gene ontology (GO) enrichment tests were used to identify specific functions showing enriched representation in regions with elevated/decreased variability or differentiation; these tests were performed using gene annotations from the Ensemble *G. aculeatus* database and the BINGO v2.44 plugin (Maere *et al.* 2005) in Cytoscape v2.8.2 (Shannon *et al.* 2003). Gene sets of different sizes were used for GO enrichment tests and were constructed by choosing the genes with the lowest *P*-values for variability and the differentiation tests. As low coverage may introduce noise in our analysis and reduce our ability to detect significantly enriched GO categories, we performed several tests for each of the three G_{st} analyses using different thresholds for the *P*-values and for the top genes cut-off values in the ranking. GO term annotation of the three-spined genes was based on the HUGO Gene Nomenclature Committee (HGNC) symbols provided in the Ensembl database. The HGNC symbols were converted to the corresponding UniProt symbols using the HGNC database and then annotated using the GO database. The GO enrichment tests were performed using a hypergeometric test and a Benjamini and Hochberg false discovery rate correction for multiple testing (Benjamini & Hochberg 1995).

Results

De novo consensus sequence assembly and SNP detection

A total of 3 010 895 forward and 3 167 130 reverse population-matched reads were produced by Illumina GA

II sequencing, with the number of reads varying more than eightfold between nine-spined stickleback populations (ranging from 78 983 in SKA to 652 455 in PYÖ; Table 1). We identified 55 193 forward and 58 900 reverse consensus sequences (69 and 68 bases long, respectively), which are predicted to cover approximately 1.7% of *P. pungitius* genome, assuming similar genome size in nine- and three-spined sticklebacks. 15 316 of the consensus sequences contained polymorphic sites used in downstream analysis (13 941 single SNPs and 1375 haplotypes segregating as SNPs) with a median read depth of 37 copies across all populations, and an estimated genome-wide average SNP density of 1.93 SNPs per kb. Consensus sequence and SNP information is contained in Table S1 (Supporting information).

Mean population variability and divergence

On the basis of analysis of all 15 316 SNPs and on smaller subsets of markers, we observed large variation in genetic diversity amongst nine-spined stickleback populations, similar to earlier microsatellite analyses (Shikano *et al.* 2010c). Marine populations (HKI, LEV) exhibited the highest diversity, whereas lake (POR, SKA) and particularly pond (PYÖ, BYN, RYT, ABB) populations showed reduced variability irrespective of diversity measure or number of SNPs used (Table 2). High correlations were observed between population diversity indices derived from genome-wide SNP data and those derived from 12 microsatellite loci (using all 15316 SNP loci, Pearson's $r = 0.97$, $P = 4 \times 10^{-5}$ for expected heterozygosities and $r = 0.88$, $P = 4 \times 10^{-3}$ for proportions of polymorphic loci; Table 3). Overall, pairwise correlations between all diversity estimators were high (Table 3). To control for the potential effect of unequal SNP coverage per populations, we performed a bootstrap analysis on SNP read data sets normalized to the lowest number of SNP reads in one population (18 943 reads, SKA population). The obtained correlations were very similar to the ones from the whole SNP data set (Pearson's $r = 0.97$, $P = 2.5 \times 10^{-5}$ for expected heterozygosities and $r = 0.88$, $P = 3.5 \times 10^{-3}$ for proportions of polymorphic loci). Genetic distance matrices built using SNPs and microsatellites were also significantly correlated (Mantel test, $Z = 3.36$, $P = 0.046$). On the other hand, when differentiation patterns among eight populations were visualized using a neighbour-joining tree, populations clustered according to their geographic origin (Baltic vs. White Sea basin) using SNPs, whereas only one node (HKI and LEV) was supported by bootstrap estimates using microsatellite loci (Fig. 2).

Mapping of consensus sequences to three-spined stickleback (Gasterosteus aculeatus) genome

A total of 54 207 (47.5%) nine-spined stickleback consensus sequences mapped to a single position in the three-spined stickleback genome. After merging partially overlapping forward and reverse pairs into single sequences, a total of 49 277 non-overlapping mapped consensus sequences were obtained (46 411 of which mapped to a known chromosomal position) and 6834 of these contained a SNP. Furthermore, 5900 (12%) of the mapped consensus sequences matched to protein fragments consistent with their genomic location. In total, 423 polymorphic markers were located in coding regions, corresponding to 271 synonymous and 152 non-synonymous substitutions in nine-spined stickleback. The mean distance between adjacent consensus sequences was 8780 bases (median 3280 bases), and the mean distance between successive polymorphic markers was 61 423 bases (median 36 381 bases). A total of 3 132 161 bases aligned to the three-spined genome, with 9% mismatches in non-coding regions and 6.4% mismatches in coding regions. Altogether, 22 858 mismatches between the two species were observed in coding regions, and 40.8% (9337) of them resulted in non-synonymous mutations (Table 4).

Table 2 Diversity indices of eight populations based on RAD and microsatellite data

Marker type	Diversity index	Habitat type: Pond				Lake		Marine	
	Population	ABB	BYN	PYÖ	RYT	POR	SKA	HKI	LEV
RAD-derived SNPs	Mean H_s (over 15 316 loci)	0.190	0.129	0.047	0.111	0.127	0.077	0.311	0.283
	Mean H_s (normalized read number)	0.181	0.120	0.047	0.110	0.119	0.077	0.298	0.271
	Proportion of polymorphic loci (over 15 318 loci)	0.398	0.295	0.116	0.193	0.249	0.116	0.630	0.523
	Proportion of polymorphic loci (normalized read number)	0.261	0.164	0.066	0.158	0.172	0.116	0.421	0.371
Microsatellites ($n = 12$)	H_e	0.264	0.130	0.004	0.178	0.215	0.155	0.590	0.530
	PIC	0.223	0.111	0.004	0.153	0.195	0.126	0.533	0.491
	H_o	0.272	0.137	0.004	0.175	0.188	0.175	0.590	0.521
	Proportion of polymorphic loci	0.750	0.583	0.167	0.417	0.833	0.417	1.000	0.917

H_s, an unbiased estimator of gene diversity (Nei & Roychoudhury 1974); H_e, expected heterozygosity; H_o, observed heterozygosity; PIC, polymorphism information content. Microsatellite data were obtained from Shikano *et al.* (2010c). Estimators calculated from normalized read number are the mean values obtained from 100 bootstraps using a subset of 18943 random reads per population.

Table 3 Correlation between RAD-derived SNPs and microsatellites diversity estimator values

Marker type	Diversity index	Mean H_s	Mean H_s (normalized)	Proportion of polymorphic loci	Proportion of polymorphic loci (normalized)	H_e	PIC	H_o	Proportion of polymorphic loci
RAD-derived SNPs	Mean H_s	1	—	—	—	—	—	—	—
	Mean H_s (normalized)	1	1	—	—	—	—	—	—
	Proportion of polymorphic loci	0.987	0.984	1	—	—	—	—	—
	Proportion of polymorphic loci (normalized)	0.998	1	0.983	1	—	—	—	—
Microsatellites ($n = 12$)	H_e	0.975	0.978	0.934	0.980	1	—	—	—
	PIC	0.974	0.977	0.932	0.977	1	1	—	—
	H_o	0.973	0.977	0.932	0.980	0.998	0.995	1	—
	Proportion of polymorphic loci	0.888	0.880	0.880	0.885	0.874	0.872	0.857	1

Pairwise Pearson's r was calculated using data from Table 2, and diversity indices are for the same data as in Table 2.

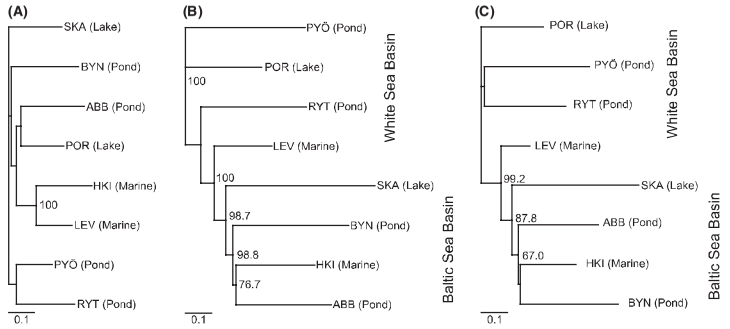


Fig. 2 Genetic relationships between populations visualized using neighbour-joining trees and Cavalli-Sforza chord distance. Relationships were determined based on (A) 12 microsatellite and insertion-deletion loci (Shikano *et al.* 2010c); (B) 3624 polymorphic RAD markers (minimum read coverage one per population); (C) 589 polymorphic RAD markers (minimum read coverage four per population). Numbers on the branches correspond to >50% bootstrap support values based on 1000 replications.

Genome-wide distribution of consensus sequences and SNPs

When using the LRM to compare between genome-wide fragment densities (at 10 752 positions throughout the genome), all slopes were highly significant ($P < 10^{-15}$). Overall, the distribution of the observed consensus sequence positions in nine-spined stickleback followed the expected distribution from *in-silico* digestion of the three-spined stickleback genome (Fig. 3A, LRM $R^2 = 0.29$). Both the expected and observed distributions showed reduced abundance of *Eco*RI-*Hae*III

Table 4 Summary of base and amino-acid changes observed between three-spined and nine-spined stickleback sequences

	Overall	Non-coding sequences	Coding sequences
Aligned bases	3 132 161	2 775 254	356 907 (118 969 amino-acids)
Mismatches	273 147	250 289	22 858
% of aligned bases	8.7	9	6.40
Synonymous base changes	—	—	13 521
Non-synonymous base changes	—	—	9337

fragments towards the chromosome ends, which was not because of an edge effect from the density estimation procedure. The distribution of the observed coding consensus sequences in nine-spined stickleback also matched the distribution of three-spined stickleback exons (Fig. 3B, LRM $R^2 = 0.49$) and followed the overall distribution of all mapped consensus sequences (LRM $R^2 = 0.25$). Similarly, the overall distribution of SNPs matched the distribution of all mapped sequences (Fig. 3C, LRM $R^2 = 0.60$) and showed low correlation when compared with the three-spined stickleback exon distribution (LRM $R^2 = 0.02$). However, several regions exhibited significantly higher or lower levels of polymorphism (i.e. deviating significantly from random distribution; Fig. 3D). Fifteen genomic regions, located on 10 chromosomes (LGs I, III, VII, VIII, IX, XIII, XVI, XVII, XIX, XX) had an excess of SNPs (kernel permutation test, $P < 0.01$, q-value < 0.37; Fig. 3D), while 24 regions on 15 chromosomes (all but LGs II, VI, XII, XV, XVIII) had reduced polymorphism (kernel permutation test, $P < 0.01$, q-value < 0.21; Fig. 3D). Genomic regions with SNP excess were significantly enriched for the GO terms representing following biological processes: immunity, lipid signalling and metabolism, taste stimulus response, axon regeneration and smooth muscle structure (Table 5). No category was significantly enriched in regions showing reduced polymorphism.

Chromosome-level differences in SNP density, divergence and variability

The number of consensus sequences mapping to each chromosome was approximately proportional to the chromosome length in the three-spined stickleback (Fig. 4A, LRM $R^2 = 0.93$). The number of polymorphic markers identified for each chromosome was also highly correlated with the chromosome length in three-spined stickleback (Fig. 4B, LRM $R^2 = 0.86$), with the notable exception of the sex-determining chromosome LG XII, which exhibited a higher number of SNPs than expected given its length in three-spined stickleback (Fig. 4B). Further investigation revealed that the distribution of SNPs along LG XII was rather uniform, without obvious differences along the chromosome (Fig. 3C). This chromosome also showed higher heterozygosity and a significantly reduced level of divergence between marine and freshwater habitats when compared with the other chromosomes (Kruskal–Wallis test on G_{st} values: $\chi^2 = 37.60$, d.f. = 20, $P = 0.01$) (Fig. 4C, D).

Genome-wide distribution of differentiation between marine and freshwater habitats

When comparing marine (HKI and LEV) and freshwater (ABB, BYN, SKA, PYÖ, RYT and POR) habitats, we identified nine regions with significantly elevated divergence on eight chromosomes (kernel permutation test, $P < 0.0025$, q-value < 0.48). In addition, eight regions with reduced G_{st} values (kernel permutation test, $P < 0.002$, q-value < 0.55) were detected on eight different chromosomes (Fig. 5). For GO enrichment analysis, we selected a less stringent significance threshold ($P < 0.01$), as possible false-positive regions are not expected to result in significant enrichment of specific biological function. Thus, GO tests indicated that genomic regions exhibiting elevated differentiation were enriched for genes with functions related to kidney development and function, immunity, and regulation of MAP kinase pathways (Table 5 and Table S2, Supporting information). On the other hand, regions that exhibited decreased differentiation between marine and freshwater populations were enriched for genes related to haemostasis, lipid signalling and metabolism, and energy reserve metabolism (Table S2, Supporting information).

Discussion

In this study, we have shown that a RAD-based genome complexity reduction protocol can be successfully used to generate a reduced representation of the nine-spined stickleback genome, with a sufficient depth of coverage not only for SNP detection, but also for preliminary estimation of population- and habitat-wide allele frequencies. Furthermore, the exploitation of synteny between nine-spined and three-spined stickleback genomes has provided a foundation for the characterization of genome-wide variability and differentiation patterns. Our study highlights a number of potential advantages over more traditional multi-step strategies, where identification of SNPs is carried out separately and followed by the development of high-throughput genotyping platforms with many individuals. In the following discussion, we describe in detail the advantages, limitations and the main molecular evolutionary and population genetic conclusions of our study, as well as the implications of the current work for future genome-wide population genetic studies in non-model species.

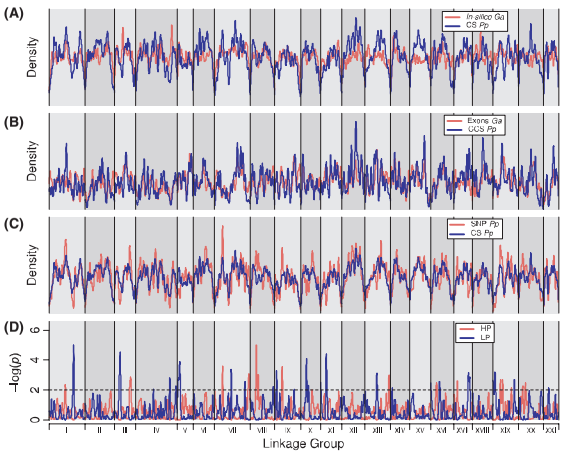


Fig. 3 Distributions of the mapped expected and observed consensus sequences using the three-spined stickleback genome as reference. (A) Distribution of expected fragments (*in-silico Ga*) and observed consensus sequences (CS Pp). Expected fragments were selected for a size range of 220–440 bases after *in-silico* digestion of the three-spined genome sequence. (B) Distribution of all known three-spined exonic sequences (exons *Ga*) and observed nine-spined consensus coding sequences (CCS *Pp*). (C) Distribution of SNPs (SNP *Pp*) compared to the distribution of consensus sequences (CS *Pp*). (D) Evidence for non-random distribution of SNPs across the genome. Red and blue lines correspond to ($-\log_{10}$) transformed *P*-values from permutation testing indicating regions of elevated (high polymorphism, HP) and reduced (low polymorphism, LP) variation, respectively.

Adaptive divergence in nine-spined stickleback

At the species level, we identified 15 and 24 chromosome regions in nine-spined stickleback with increased and reduced levels of polymorphism which may correspond to areas affected by balancing and directional selection, respectively. GO analysis revealed that regions with an excess of SNPs are enriched for genes involved in multiple functions such as immunity, chemical stimulus response, lipid metabolism and signalling pathways (Table S3, Supporting information). However, caution is warranted when interpreting GO enrichment tests in the context of genome scans, as genes with similar function sometimes cluster to specific parts of the genome, such as with olfactory receptor genes in the human genome (Niimura & Nei 2005). As a result, significant GO categories associated with multiple distinct outlier regions (such as immune function, detection of chemical stimuli, contractile fibre and hexose metabolic process) are more likely to indicate regions where selection is targeting genes with a specific function than GO categories associated with a single outlier region (Table 5). There was no obvious correlation between gene density (*i.e.* gene deserts or gene rich regions) and exceptionally high or low variability.

When comparing marine and freshwater population groups, we identified nine genomic regions with elevated levels of differentiation (permutation testing, $P < 0.0025$, q-value < 0.48), which are potentially affected by divergent selection between habitats. Subsequent GO tests showed that regions of elevated differentiation were enriched for several functions related to osmoregulation, such as kidney development and positive regulation of the extracellular signal-regulated kinase ERK1 and ERK2 cascade. ERK1 and ERK2 are MAP kinases involved in the response to change in salinity (Kültz 2001; Madsen *et al.* 2007), but may have various other important roles in synaptic plasticity (Thomas & Huganir 2004; Leal *et al.* 2006) and muscle response to exercise (Widegren *et al.* 2000). The nine-spined stickleback populations studied here differ in their brain anatomy and plasticity (Gonda *et al.* 2009,

Table 5 Significantly enriched Gene Ontology (GO) categories in genes exhibiting high G_{st} values, low G_{st} values or high polymorphism levels. No category was significantly enriched in genes exhibiting low polymorphism levels. GO categories for each function are sorted by decreasing order of evidence, based on the GO enrichment test *P*-value and the number of distinct genomic regions involved

Candidate regions	Global function	GO category	GO enrichment test *P*-value	Number of genes involved	Chromosomes
SNP excess	Glycoside metabolism	Hexose metabolic process	1.26×10^{-4}	30	II, III, V, VI, VII, X, XIII, XVI, XVII, XVIII, XIX, XX
		Glucose metabolic process	6.21×10^{-3}	21	III, V, VI, VII, X, XIII, XVI, XVII, XVIII, XIX, XX
	Smooth muscle-related	Contractile fibre	0.035	19	I, II, III, V, VII, VIII, IX, XI, XVI, XVII, XIX, XX
		Regulation of smooth muscle cell-matrix adhesion	6.62×10^{-5}	4	III
	Chemical stimulus response	Detection of stimulus	0.019	18	II, III, VI, IX, X, XIII, XIX, XX
		Sweet taste receptor activity	1.73×10^{-3}	6	XII
	Immunity	Cytokine production	0.015	5	III, VIII, XIX
		Regulation of T-cell migration	1.15×10^{-3}	5	III, XIX
	Lipid metabolism and signalling pathways	Sterol binding	7.10×10^{-4}	5	III, IX
		Lipid transporter activity	0.045	4	III, VIII
		Cholesterol binding	4.47×10^{-3}	4	III
		Regulation of lipoprotein metabolic process	5.83×10^{-4}	4	III
		Regulation of platelet-derived growth factor receptor signalling pathway	7.10×10^{-4}	4	III
	Nervous system-related	Peripheral nervous system axon regeneration	0.042	5	III, VI
		Axon regeneration	7.63×10^{-3}	4	III
Elevated G_{st}	Kidney development	Metanephric mesenchyme development	0.037	5	I, XV, XVIII, XIX
		Glomerular capillary formation	0.023	2	IV, XVIII
		Renal sodium ion absorption	0.05	1	XVIII
	Immunity	Immune system development	0.029	7	IV, XVII, XVIII
		Positive regulation of T-cell differentiation	0.039	3	IV, XVII, XVIII
		Cellular response to macrophage colony-stimulating factor stimulus	0.02	2	IV, XVII
		Cytokine binding	0.038	3	IV, XVI
	MAP kinase regulation	Regulation of protein modification process	0.046	8	IV, XVII, XVIII
		Positive regulation of kinase activity	0.05	5	IV, XVII
		Positive regulation of ERK1 and ERK2 cascade	0.033	3	IV

continued

Table 5 *continued*

Candidate regions	Global function	GO category	GO enrichment test *P*-value	Number of genes involved	Chromosomes
Reduced G_{st}	Haemostasis	Blood coagulation	0.039	10	IV, XII, XVIII, XIX, XX
		Artery morphogenesis	0.046	3	XVIII
	Energy reserve metabolism	Energy reserve metabolic process	0.036	6	V, XI, XII, XVIII
	Lipid signalling and metabolism	Regulation of lipid biosynthetic process	0.01	5	V, XVIII, XIX
		Cholesterol homoeostasis	2.99×10^{-3}	5	V, XVIII
		Response to inorganic substance	0.016	8	XVIII, XIX
		Regulation of steroid metabolic process	0.015	4	XVIII, XIX
		Cellular response to prostaglandin stimulus	6.98×10^{-4}	3	XVIII

2012) and in their behaviour (Herczeg *et al.* 2009a; Herczeg & Välimäki 2011). Therefore, the MAP kinase regulatory pathways are good candidates for potential long-range physiological effects in these adaptive traits. The comparison of marine and freshwater populations also revealed eight genomic regions showing reduced levels of differentiation ($P < 0.002$, q-value < 0.55). In these regions, GO tests indicated that putative candidates for balancing selection are enriched for genes involved in blood coagulation and lipid signalling. This included prostaglandin, which is involved in haemostasis (Stenslokken *et al.* 2006), osmoregulation (Brown *et al.* 1991) and glucose metabolism regulation (Busby *et al.* 2002).

In this study, none of the regions with unusual patterns of variability or differentiation between habitat types in the nine-spined stickleback corresponded to the candidate regions for adaptive divergence between oceanic and freshwater three-spined sticklebacks reported in previous studies (*e.g.* Hohenlohe *et al.* 2010; DeFaveri *et al.* 2011). This finding is not totally unexpected for several reasons (*e.g.* Akey 2009; Kaeuffer *et al.* 2012). First, different founding populations may differ in genetic architecture for a particular trait (e.g. Simoes *et al.* 2008). Second, similar phenotypic changes may occur because of mutations of different genes (e.g. Steiner *et al.* 2009). Finally, genetic architecture of several traits, such as sex, lateral plate number and reduction of the pelvic skeleton, map to different chromosomes in nine-spined and three-spined stickleback (Colosimo *et al.* 2004; Peichel *et al.* 2004; Shapiro *et al.* 2009). Therefore, it is possible that much of the adaptive divergence and similar phenotypic traits in these fish species that diverged more than 13 million years ago (Bell *et al.* 2009) may have different genetic origins (Shapiro *et al.* 2009). Moreover, genetic architecture of the same traits may also vary within species, as we found no elevated differentiation between habitat types in regions implicated in freshwater adaptation in other marine and freshwater populations of nine-spined stickleback across Fennoscandia, as identified by Shikano *et al.* (2010c). Taken together, it appears that although parallel or convergent evolution has often been reported, it is certainly not pervasive (Kaeuffer *et al.* 2012 and references therein), and several genetic, ecological, functional and sexual factors may be responsible for genotypic and phenotypic non-parallelism. Therefore, identified regions contain promising candidate genes that may have been affected by selection, but additional analyses of individual populations with much higher coverage is required to provide further insights into parallel vs. non-parallel genetic changes, and to further strengthen the evidence of non-neutral evolution.

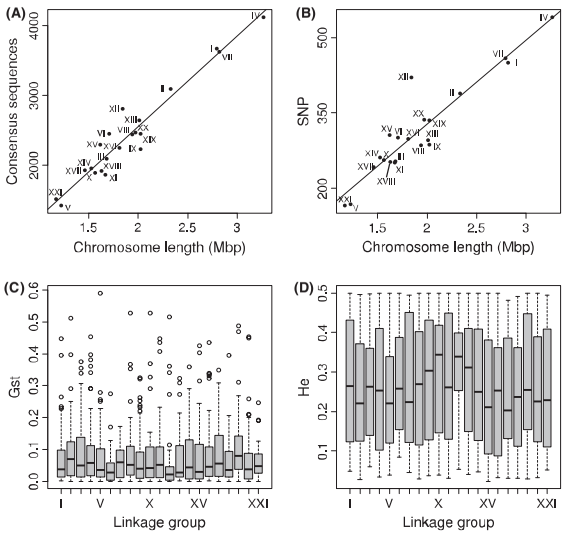


Fig. 4 Chromosome-level differences in SNP density, divergence and variability. Number of (A) consensus sequences and (B) polymorphic markers, mapped to each chromosome. Boxplots of (C) G_{st} values and (D) expected heterozygosity for each chromosome (minimum coverage per habitat of 20 reads).

Genetic characterization of the sex chromosome, LG XII

Our chromosome-level analysis of SNP distribution, divergence and variability revealed marked differences between the nascent sex-determining chromosome LG XII and all other chromosomes. In LG XII, we observed higher SNP density and heterozygosity, as well as reduced differentiation between freshwater and marine populations. We suggest that these observations can be explained by two factors: first, our pooled samples represent a mixture of both males and females sampled from the same population; and second, nine-spined sticklebacks have shown a high degree of differentiation and fixation of alleles between nascent X and Y chromosomes (Shikano *et al.* 2011). As a result, it is highly likely that a large proportion of the identified SNPs corresponding to LG XII represent alternative alleles from divergent X and Y chromosomes, rather than true autosomal markers following Mendelian segregation. We found that the distribution of identified variants along XII was rather uniform, which is consistent with a high level of divergence between X and Y chromosomes throughout LG XII, a pattern also observed earlier using 14 sex-linked microsatellite markers (Shikano *et al.* 2011). Overall, these results demonstrate the utility and potential of RAD sequence analysis in sex chromosome evolution, providing novel insights into sex-specific patterns of genetic variability.

Advantages of the paired-end double-RAD approach (PE dRAD)

In non-model species, the use of paired-end sequencing has an advantage over the use of single reads alone, as it provides information from longer contiguous DNA fragments, from which primers for subsequent SNP validation or/and targeted genotyping can be developed. Previous studies (*e.g.* Baird *et al.* 2008; Etter *et al.* 2011) relied on physical shearing of DNA after ligation of the first adapter, producing fully overlapping forward reads, but only partially overlapping reverse reads. This method is useful for the assembly of longer contiguous sequences, but at cost of reduced coverage in reverse reads in comparison with the forward reads. Therefore, rather than carrying out physical shearing of double-stranded DNA, we instead used a second restriction enzyme with relatively frequent restriction sites throughout the genome (*Hae*III) to create fully overlapping forward and reverse reads. Physical shearing requires substantial 'hands on' time and specialized equipment; therefore, adding the second enzymatic reaction step provides a simpler and faster method for generating fully overlapping sequences for both forward and

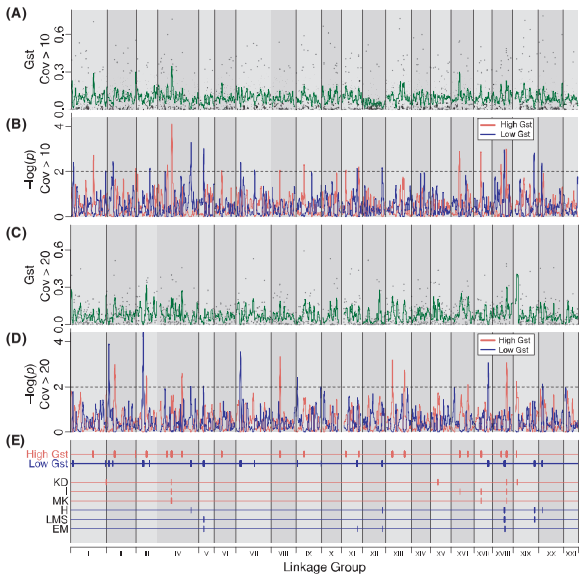


Fig. 5 Differentiation between the genomes of nine-spined sticklebacks from marine and freshwater habitats. (A) Smoothed G_{st} profile (green line) using a minimum coverage threshold of 10 per habitat; the black dots correspond to the original G_{st} values for individual markers. (B) *P*-values of the G_{st} profile. These were obtained from permutation testing (transformed to $-\log_{10}P$) for elevated differentiation (red line) and reduced differentiation (blue line) between the habitats. The dashed line indicates $P = 0.01$. (C) and (D): As for (A) and (B), but using a minimum coverage threshold of 20 per habitat. (E) Summary of genomic locations of interest. High G_{st} lane: regions with significantly elevated G_{st} ($P < 0.01$ for at least one G_{st} analysis). Low G_{st} lane: regions with significantly reduced G_{st} value ($P < 0.01$). Additional lanes location of genes with significant GO terms; KD, kidney development; I, immunity; MK, MAP kinases; H, haemostasis; LMS, lipid metabolism and signalling; EM, energy metabolism (Table 5).

reverse reads. This modification is in line with the development of faster, flexible and inexpensive methods that simplify and streamline genome complexity reduction procedures (for example, see Elshire *et al.* 2011).

Allelotyping-by-sequencing and SNP validation

We have shown that DNA pooling is a cost-effective approach for SNP discovery and allele frequency estimation at a population-wide level. This method avoids redundant sequences associated with individual genotyping and allows rapid generation of data required for population genetic studies at the genome-wide level (Futschik & Schlötterer 2010). However, we acknowledge several limitations associated with the allelotyping approach. First, the accuracy of the pooling approach for allele frequency estimation is likely to be influenced by individual DNA concentration and quality, size of the DNA pool, variation in amplification during sequencing and technical differences between sequencing lanes (discussed in Cutler & Jensen 2010 and Mullen *et al.* 2012). Second, DNA pooling results in loss of haplotype information (*e.g.* linkage disequilibrium), but this is outweighed by the reduced cost and increased accuracy in population genetic inference (Futschik & Schlötterer 2010). Finally, stringent quality control means that rare allelic variants may not be detected; this is particularly relevant to the current study, where at least three copies of an allele would have to be present for use in further analysis. Nevertheless, our results are in line with other studies that suggest that allelotyping of pooled DNA is a powerful tool for genome-wide characterization of variability and differentiation both in model and in non-model species (van Tassell *et al.* 2008; Futschik & Schlötterer 2010; Mullen *et al.* 2012).

One drawback of this study is that allele frequency estimates were not validated using a subset of loci. This is usually carried out in studies that aim to identify

single SNPs that strongly associate with the phenotype of interest (*e.g.* van Tassell *et al.* 2008; Mullen *et al.* 2012). However, the primary purpose of this study was not to identify single outlier loci that deviate from the neutrality, but rather to test a modified genomic complexity reduction method and characterize a significant proportion of the nine-spined stickleback genome. Furthermore, we observed strikingly high correlations (Pearson's $r > 0.88$) when comparing diversity indices from allele frequencies estimated in this study and from 12 previously published microsatellite loci (Shikano *et al.* 2010c). Similarly high correlations were observed when bootstrap analyses were performed on SNP read data sets normalized to the lower number of SNP reads observed in one population (18 943 reads, SKA population). Overall, pairwise correlations between all diversity estimators were high (Table 3). This indicates that our genome-wide inferences from pooled DNA are likely to reflect the underlying population genetic patterns. However, genetic relationships between eight populations showed somewhat different patterns between SNPs and microsatellites. We suggest that this discrepancy is most likely explained by the combination of relatively low power of 12 microsatellite loci and strong effect of genetic drift in freshwater populations causing erroneous clustering of the two most polymorphic marine populations (HKI and LEV; Fig. 2A). The current SNP data set grouped populations together based on drainage, suggesting the genome-wide markers correctly reflect the post-glacial origin of populations (Fig. 2B, C).

Using a closely related genome as a reference for a non-model species

At a genome-wide level, chromosomal rearrangements and displacements between three-spined and nine-spined stickleback genomes may introduce a source of uncertainty in the estimated parameters. However, any rearrangement of large blocks of the genome would only introduce uncertainty at the boundaries of such rearrangements, with little influence on the information contained within such blocks. By using a kernel smoothing approach, we aimed to identify genomic regions, rather than single loci that differ markedly from the rest of the genome. This approach both reduces the noise of individual G_{st} values and enables identification of regions that significantly differ from the neighbouring areas via permutation testing, thus taking the differences in marker density into account and increasing the statistical power for detecting outlier regions when several markers with consistent G_{st} values are clustered. Furthermore, the high correlation between expected and observed distributions of mapped RAD fragments (Fig. 3), as well as between chromosome length and the number of aligned consensus sequences (Fig. 4A), suggests that we can confidently use the three-spined stickleback genome as a reference for genomic analysis of nine-spined stickleback. From our comparative data (Table 4), about 39 Mb (8.7%) is expected to differ between the two species genomes for an estimated genome size of 446 Mb, resulting in a 7.8% divergence between the protein amino-acid sequences. This is in the range of what is observed from previous studies for nucleotide divergence (3.2% and 13% for nuclear and mitochondrial sequences, respectively), but is higher than estimates for protein divergence (1.7% and 3.8% divergence for nuclear and mitochondrial encoded proteins, respectively; Kawahara *et al.* 2009).

Conclusions

We have described the development of a novel genomic complexity reduction protocol and combined next generation sequencing technology with a cost-effective DNA pooling strategy to characterize a significant proportion of the nine-spined stickleback genome. We increased the genomic resources available for nine-spined stickleback from several hundred nuclear and mitochondrial DNA sequences, to more than 114 000 consensus sequences, of which more than 15 000 contain polymorphic SNPs. As such, this work creates a valuable genomic resource for future genetic studies of the nine-spined stickleback. This work also demonstrates how the combination of inter- and intra-specific RAD analysis can reveal novel genome-wide patterns of adaptation in a non-model species.

Acknowledgements

We thank Victor Berger, Göran Engström, Abigel Gonda, Pirkko Siikamäki, the Oulanka Research Station (University of Oulu) and the White Sea Biological Station (Russian Academy of Sciences) for help in obtaining the samples. We also thank Takahito Shikano for sharing microsatellite data, and Spiros Papakostas, Erica Leder, Heidi Viitaniemi and Shihab Hasan for discussions regarding the analyses. Illumina sequencing was overseen by Pekka Ellonen at the Institute for Molecular Medicine, Finland. Our research was supported by the Academy of Finland (grant numbers 34728, 250435 to JM, 133875, 141231 to CRP and 128716 to GH) and Estonian Science Foundation (grant numbers 6802, 8215 to AV).

References

Akey JM (2009) Constructing genomic maps of positive selection in humans: where do we go from here? *Genome Research*, **19**, 711–722.

Aljanabi SM, Martinez I (1997) Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Research*, **25**, 4692–4693.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403–410.

Baird NA, Etter PD, Atwood TS, *et al.* (2008) Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PLoS One*, **3**, e3376.

Baxter SW, Davey JW, Johnston JS, Shelton AM, Heckel DG, Jiggins CD, Blaxter ML (2011) Linkage mapping and comparative genomics using next-generation RAD sequencing of a non-model organism. *PLoS One*, **6**, e19315.

Bell MA, Stewart JD, Park PJ (2009) The world's oldest fossil three-spined stickleback fish. *Copeia*, **2009**, 256–265.

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate – a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B (Statistical Methodology)*, **1**, 289–300.

Brown JA, Gray CJ, Hattersley G, Robinson J (1991) Prostaglandins in the kidney, urinary bladder and gills of the rainbow trout and European eel adapted to fresh water and seawater. *General and Comparative Endocrinology*, **84**, 328–335.

Busby ER, Cooper GA, Mommsen TP (2002) Novel role for prostaglandin E2 in fish hepatocytes: regulation of glucose metabolism. *Journal of Endocrinology*, **174**, 137–146.

Cavalli-Sforza LL, Edwards AWF (1967) Phylogenetic analysis: models and estimation procedures. *American Journal of Human Genetics*, **19**, 233–257.

Chen T-R, Reisman HM (1970) A comparative chromosome study of the North American species of sticklebacks (Teleostei: Gasterosteidae). *Cytogenetics*, **9**, 321–332.

Colosimo PF, Peichel CL, Nereng K *et al.* (2004) The genetic architecture of parallel armor plate reduction in three-spined sticklebacks. *PLoS Biology*, **2**, e109.

Coyle SM, Huntingford FA, Peichel CL (2007) Parallel evolution of *Pitx1* underlies pelvic reduction in Scottish three-spined stickleback (*Gasterosteus aculeatus*). *Journal of Heredity*, **98**, 581–586.

Cresko WA, Amores A, Wilson C *et al.* (2004) Parallel genetic basis for repeated evolution of armor loss in Alaskan three-spined stickleback populations. *Proceedings of the National Academy of Sciences USA*, **101**, 6050–6055.

Cutler DJ, Jensen JD (2010) To pool, or not to pool? *Genetics*, **186**, 41–43.

Deagle BE, Jones FC, Chan YF *et al.* (2011) Population genomics of parallel phenotypic evolution in stickleback across stream-lake ecological transitions. *Proceedings of the Royal Society of London Series B: Biological Sciences*, **279**, 1277–1286.

DeFaveri J, Shikano T, Shimada Y, Goto A, Merilä J (2011) Global analysis of genes involved in freshwater adaptation in threespine sticklebacks (*Gasterosteus aculeatus*). *Evolution*, **65**, 1800–1807.

Ekblom R, Galindo J (2011) Applications of next generation sequence in molecular ecology of non-model organisms. *Heredity*, **107**, 1–15.

Ellegren H, Sheldon BC (2008) Genetic basis of fitness differences in natural populations. *Nature*, **452**, 169–175.

Elphinstone MS, Hinten GN, Anderson MJ, Nock CJ (2003) An inexpensive and high-throughput procedure to extract and purify total genomic DNA for population studies. *Molecular Ecology Notes*, **3**, 317–320.

Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K *et al.* (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS One*, **6**, e19379.

Etter PD, Preston JL, Bassham S, Cresko WA, Johnson EA (2011) Local de novo assembly of RAD paired-end contigs using short sequencing reads. *PLoS One*, **6**, e18561.

Feder ME, Mitchell-Olds T (2003) Evolutionary and ecological functional genomics. *Nature Reviews Genetics*, **4**, 649–655.

Felsenstein J (2004) PHYLIP (Phylogeny Inference Package) Version 3.6. Department of Genome Sciences, University of Washington, Seattle, Washington.

Flicek P, Amode MR, Barrell D, Beal K *et al.* (2011) Ensembl 2011. *Nucleic Acids Research*, **39**, Database issue, D800-D806

Flori L, Fritz S, Jaffrézic F *et al.* (2009) The genome response to artificial selection: a case study in dairy cattle. *PLoS One*, **4**, e6595.

Fournier-Level A, Korte A, Cooper MD, Nordborg M, Schmitt J, Wilczek AM (2011) A map of local adaptation in *Arabidopsis thaliana*. *Science*, **334**, 86–89.

Futschik A, Schlötterer C (2010) The next generation of molecular markers from massively parallel sequencing of pooled DNA samples. *Genetics*, **186**, 207–218.

Goecks J, Nekrutenko A, Taylor J (2010) Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biology*, **11**, R86.

Gonda A, Herczeg G, Merilä J (2009) Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? *Journal of Evolutionary Biology*, **22**, 1721–1726.

Gonda A, Välimäki K, Herczeg G, Merilä J (2012) Brain development and predation: plastic responses depend on evolutionary history. *Biology Letters*, **26**, 249–252.

Herczeg G, Välimäki K (2011) Intraspecific variation in behaviour: effects of evolutionary history, ontogenetic experience and sex. *Journal of Evolutionary Biology*, **24**, 2434–2444.

Herczeg G, Gonda A, Merilä J (2009a) Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *Journal of Evolutionary Biology*, **22**, 544–552.

Herczeg G, Gonda A, Merilä J (2009b) Evolution of gigantism in nine-spined stickleback. *Evolution*, **63**, 3190–3200.

Herczeg G, Turtiainen M, Merilä J (2010) Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. *Biological Journal of the Linnean Society*, **101**, 403–416.

Herczeg G, Gonda A, Kuparinen A, Merilä J (2012) Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? *Evolutionary Ecology*, **26**, 109–122.

Hohenlohe PA, Bassham S, Etter PD, Stiffler N, Johnson EA, Cresko WA (2010) Population genomics of parallel adaptation in three-spined stickleback using sequenced RAD tags. *PLoS Genetics*, **6**, e1000862.

Hohenlohe PA, Amish S, Catchen JM, Allendorf FW, Luikart G (2011) RAD sequencing identifies thousands of SNPs for assessing hybridization between rainbow trout and westslope cutthroat trout. *Molecular Ecology Resources*, **11**, 117–122.

Huang X, Madan A (1999) CAP3: a DNA sequence assembly program. *Genome Research*, **9**, 868–877.
Jelier R, Semple JI, Garcia-Verdugo R, Lehner B (2011) Predicting phenotypic variation in yeast from individual genome sequences. *Nature Genetics*, **43**, 1270–1274.
Jones FC, Grabherr MG, Chan YF, Russell P, Mauceli E *et al.* (2012) The genomic basis of adaptive evolution in threespine sticklebacks. *Nature*, **484**, 55–61.
Kaeuffer R, Peichel CL, Bolnick DI, Hendry AP (2012) Parallel and nonparallel aspects of ecological, phenotypic, and genetic divergence across replicate population pairs of lake and stream stickleback. *Evolution*, **66**, 402–418.
Kawahara R, Miya M, Mabuchi K, Near TJ, Nishida M (2009) Stickleback phylogenies resolved: evidence from mitochondrial genomes and 11 nuclear genes. *Molecular Phylogenetics and Evolution*, **50**, 401–404.
Kofler R, Schlötterer C, Lelley T (2007) SciRoKo: a new tool for whole genome microsatellite search and investigation. *Bioinformatics*, **23**, 1683–1685.
Kültz D (2001) Evolution of osmosensory MAP kinase signaling pathways. *American Zoologist*, **41**, 743–757.
Leal RB, Ribeiro SJ, Posser T *et al.* (2006) Modulation of *ERK1/2* and p38(*MAPK*) by lead in the cerebellum of Brazilian catfish *Rhamdia quelen*. *Aquatic Toxicology*, **77**, 98–104.
Li H, Ruan J, Durbin R (2008) Mapping short DNA sequencing reads and calling variants using mapping quality scores. *Genome Research*, **18**, 1851–1858.
Madsen SS, Jensen LN, Tipsmark CK, Kiilerich P, Borski RJ (2007) Differential regulation of cystic fibrosis transmembrane conductance regulator and Na^+,K^+ -ATPase in gills of striped bass, *Morone saxatilis*: effect of salinity and hormones. *Journal of Endocrinology*, **192**, 249–260.
Maere S, Heymans K, Kuiper M (2005) BiNGO: a cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics*, **21**, 3448–3449.
Miller MR, Brunelli JP, Wheeler PA *et al.* (2012) A conserved haplotype controls parallel adaptation in geographically distant salmonid populations. *Molecular Ecology*, **21**, 237–249.
Mullen MP, Creevey CJ, Berry DP *et al.* (2012) Polymorphism discovery and allele frequency estimation using high-throughput DNA sequencing of target-enriched pooled DNA samples. *BMC Genomics*, **13**, 16.
Nei M, Roychoudhury AK (1974) Sampling variances of heterozygosity and genetic distance. *Genetics*, **76**, 379–390.
Nielsen R, Paul JS, Albrechtsen A, Song YS (2011) Genotype and SNP calling from next-generation sequencing data. *Nature Reviews Genetics*, **12**, 443–451.
Niimura Y, Nei M (2005) Comparative evolutionary analysis of olfactory receptor gene clusters between humans and mice. *Gene*, **346**, 13–21.
Papakostas S, Vasemägi A, Vähä J-P, Himberg M, Peil L, Primmer CR (2012) A proteomics approach reveals divergent molecular responses to salinity in populations of European whitefish (*Coregonus lavaretus*). *Molecular Ecology*, **21**, 3516–3530.
Peichel CL, Ross JA, Matson CK *et al.* (2004) The master sex-determination locus in three-spined sticklebacks is on a nascent Y chromosome. *Current Biology*, **14**, 1416–1424.
de Ridder J, Uren A, Kool J, Reinders M, Wessels L (2006) Detecting statistically significant common insertion sites in retroviral insertional mutagenesis screens. *PLoS Computational Biology*, **2**, e166.
Roesti M, Hendry AP, Salzburger W, Berner D (2012) Genome divergence during evolutionary diversification as revealed in replicate lake–stream stickleback population pairs. *Molecular Ecology*, in press, doi: 10.1111/j.1365-294X.2012.05509.x
Scaglione D, Acquadro A, Portis E, Tirone M, Knapp SJ, Lanteri S (2012) RAD tag sequencing as a source of SNP markers in *Cynara cardunculus L.* *BMC Genomics*, **13**, 3.
Shahin A, van Gurp T, Peters SA, Visser RGF, van Tuyl JM, Arens P (2012) SNP markers retrieval for a non-model species: a practical approach. *BMS Research Notes*, **5**, 79.
Shannon P, Markiel A, Ozier O *et al.* (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Research*, **13**, 2498–2504.
Shapiro MD, Marks ME, Peichel CL *et al.* (2004) Genetic and developmental basis of evolutionary pelvic reduction in three-spined sticklebacks. *Nature*, **428**, 717–723.
Shapiro MD, Summers BR, Balabhadra S *et al.* (2009) The genetic architecture of skeletal convergence and sex determination in nine-spined sticklebacks. *Current Biology*, **19**, 1140–1145.
Shikano T, Jetty R, Shimada Y, Merilä J (2010a) Utility of sequenced genomes for microsatellite marker development in non-model organisms: a case study of functionally important genes in nine-spined sticklebacks (*Pungitius pungitius*). *BMC Genomics*, **11**, 334.
Shikano T, Ramadevi J, Merilä J (2010b) Identification of local- and habitat-dependent selection: scanning functionally important genes in nine-spined sticklebacks (*Pungitius pungitius*). *Molecular Biology and Evolution*, **27**, 2775–2789.
Shikano T, Shimada Y, Herczeg G, Merilä J (2010c) History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. *Molecular Ecology*, **19**, 1147–1161.
Shikano T, Natri HM, Shimada Y, Merilä J (2011) High degree of sex chromosome differentiation in stickleback fishes. *BMC Genomics*, **12**, 474.
Shimada Y, Shikano T, Kuparinen A, Gonda A, Leinonen T, Merilä J (2011) Quantitative genetics of body size and timing of maturation in two nine-spined stickleback (*Pungitius pungitius*) populations. *PLoS One*, **6**, e28859.
Simoes P, Santos J, Fragata I, Mueller LD, Rose MR, Matos M (2008) How repeatable is adaptive evolution? The role of geographical origin and founder effects in laboratory adaptation *Evolution*, **62**, 1817–1829.
Steiner CC, Rompler H, Boettger LM, Schoneberg T, Hoekstra HE (2009) The genetic basis of phenotypic convergence in beach mice: similar pigment patterns but different genes. *Molecular Biology and Evolution*, **26**, 35–45.
Stemshorn KC, Nolte AW, Tautz D (2005) A genetic map of *Cottus gobio* (Pisces, Teleostei) based on microsatellites can be linked to the physical map of *Tetraodon nigroviridis*. *Journal of Evolutionary Biology*, **18**, 1619–1624.
Stensløkken KO, Sundin L, Nilsson G (2006) Cardiovascular effects of prostaglandin F2a and prostaglandin E2 in Atlantic cod (*Gadus morhua*). *Journal of Comparative Physiology B*, **172**, 363–369.
Storey JD, Taylor JE, Siegmund D (2004) Strong control, conservative point estimation, and simultaneous conservative consistency of false discovery rates: a unified approach. *Journal of the Royal Statistical Society: Series B (Statistical Methodology)*, **66**, 187–205.
van Straalen NI, Roelofs D (2006) Introduction to Ecological Genetics. Oxford University Press, New York.
van Tassell CP, Smith TPL, Matukumalli LK *et al.* (2008) SNP discovery and allele frequency estimation by deep sequencing of reduced representation libraries. *Nature Methods*, **5**, 247–252.
Tautz D, Ellegren H, Weigel D (2010) Next generation molecular ecology. *Molecular Ecology*, **19**, 1–3.
Teacher AGF, Shikano T, Karjalainen ME, Merilä J (2011) Phylogeography and genetic structuring of European nine-spined sticklebacks (*Pungitius pungitius*) – mitochondrial DNA evidence. *PLoS One*, **6**, e19476.
Thomas GM, Huganir RL (2004) MAPK cascade signalling and synaptic plasticity. *Nature Reviews Neuroscience*, **5**, 173–183.
Ungerer MC, Johnson LC, Herman MA (2008) Ecological genomics: understanding gene and genome function in the natural environment. *Heredity*, **100**, 178–183.
Vera JC, Wheat CW, Fescemyer HW *et al.* (2008) Rapid transcriptome characterization for a nonmodel organism using 454 pyrosequencing. *Molecular Ecology*, **17**, 1636–1647.
Widegren U, Wretman C, Lionikas A, Hedin G, Henriksson J (2000) Influence of exercise intensity on ERK/MAP kinase signalling in human skeletal muscle. *European Journal of Physiology*, **441**, 317–322.

G.H. and J.M. organized collection of samples, A.V. performed the laboratory work and A.V., C.R.P. and J.M. designed the study. S.E.J., M.B. and A.V. analysed the data and wrote the first version of the article. All authors contributed significantly to revisions.

Data accessibility

Raw reads from Illumina Sequencing have been submitted to the Dryad data archive, doi: 10.5061/dryad.44sf1. Consensus DNA sequences from the Illumina read assembly, population-wide SNP counts (based on aligned reads) and BLAST results are uploaded as Supporting information (Table S1).

Supporting information

Additional Supporting Information may be found in the online version of this article.

Table S1 Raw and mapped consensus sequences with their population-wide SNP counts and BLAST results.

Table S2 Detailed descriptions of significance levels of genes involved in the enriched Gene Ontology (GO) categories described in Table 4.

Table S3 Estimated polymorphism, G_{st} and significance values for all genes assigned to chromosomal linkage groups in the Ensemble database.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

doi:10.1111/j.1420-9101.2009.01782.x

Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)?

A. GONDA, G. HERCZEG & J. MERILÄ

Ecological Genetics Research Unit, Department of Biological and Environmental Sciences, University of Helsinki, Helsinki, Finland

Keywords:

brain size;
evolution;
population differentiation;
predation;
Pungitius;
stickleback.

Abstract

Most studies seeking to provide evolutionary explanations for brain size variability have relied on interspecific comparisons, while intraspecific studies utilizing ecologically divergent populations to this effect are rare. We investigated the brain size and structure of first-generation laboratory-bred nine-spined sticklebacks (*Pungitius pungitius*) from four geographically and genetically isolated populations originating from markedly different habitats. We found that the relative size of bulbus olfactorius and telencephalon was significantly larger in marine than in pond populations. Significant, but habitat-independent population differences were also found in relative brain and cerebellum sizes. The consistent, habitat-specific differences in the relative size of bulbus olfactorius and telencephalon suggest their adaptive reduction in response to reduced (biotic and abiotic) habitat complexity in pond environments. In general, the results suggest that genetically based brain size and structure differences can evolve relatively rapidly and in repeatable fashion with respect to habitat structure.

Introduction

Large variation in brain size or structure has been reported in a number of taxa (e.g. Harvey *et al.*, 1980; Kotrschal *et al.*, 1998; Day *et al.*, 2005). Most of these studies have looked for interspecific correlations between brain architecture and various ecological factors and/or behavioural and life-history traits correlated with fitness (e.g. Garamszegi & Eens, 2004a; Sol *et al.*, 2008). For instance, brain size correlates positively with habitat complexity (Pollen *et al.*, 2007) and with social complexity and diet in cichlids (Gonzalez-Voyer *et al.*, 2009) and with bower complexity in bowerbirds (Madden, 2001). Furthermore, negative correlations among brain size and size of other organs (Aiello & Wheele, 1995; Kaufman, 2003; Pitnick *et al.*, 2006) have also been reported. However, contradictory results across higher taxa are common. For instance, forebrain size correlates positively with habitat complexity both in fishes and in primates (Clutton-Brock & Harvey, 1980; Huber *et al.*, 1997), whereas establishment success in novel environments correlates positively with brain size in birds (Sol *et al.*, 2005) but not in fishes (Drake, 2007).

Despite some criticism (Healy & Rowe, 2007), interspecific correlative studies form the cornerstone of our understanding of brain size evolution. However, intraspecific studies could provide additional and more fine-tuned means to test ideas and observations born out of interspecific studies. First, comparisons of brain size/structure differences among populations of the same species inhabiting different selective environments could provide explicit means to differentiate between different microevolutionary processes, such as natural selection and genetic drift (e.g. Merilä & Crnokrak, 2001), as causes of observed differentiation. Second, population level differences can be very informative: compared to species, populations are more likely to be found in the selective habitats where the observed differences have evolved.

Besides the well-described plasticity in brain size and structure in response to environmental differences (e.g. Maguire *et al.*, 2000; Tramontin & Brenowitz, 2000; Kihslinger & Nevitt, 2006), most intraspecific population comparisons made so far have been based on wild caught individuals (Garamszegi & Eens, 2004b; Karlen & Krubitzer, 2006). However, one study, based on laboratory lines of the medaka (*Oryzias latipes*) has reported genetically based intraspecific variation in brain size and architecture (Ishikawa *et al.*, 1999), whereas another study found genetically based brain structure differences in certain brain parts between laboratory-reared populations of guppies (*Poecilia reticulata*) originating from two rivers (Burns & Rodd, 2008). Hence, although intraspecific variation in brain size has often been found, very little is still known about genetic and adaptive basis of population differences in relative brain size and size of different parts.

Nine-spined sticklebacks (*Pungitius pungitius*) provide an excellent model for intraspecific comparisons, as they occupy markedly different habitats ranging from marine environments through large lakes to small, isolated ponds, where they are often the only fish species present (e.g. Bănărescu & Paepke, 2001). Hence, large differences can be found both in biotic (e.g. diversity of prey, competitors and predators) and abiotic (e.g. habitat structure) habitat components.

The aim of this study was to test whether differences in ecological conditions, expected to select for differences in brain morphology, have resulted in divergence of the relative brain size and structure in nine-spined sticklebacks. We predicted that fish from ponds, in the absence of predatory fish, under no or weak interspecific competition, and living in habitats with negligible structural heterogeneity have evolved relatively smaller brains or brain parts related to memory and learning compared with their conspecifics in the sea. For these purposes, we reared laboratory-born fish from two marine and two pond populations in common garden settings until they reached adult size. As far as we are aware, this is the first attempt to test for genetically based habitat-specific population differentiation in brain size and brain architecture from the wild.

Correspondence: Abigél Gonda, Ecological Genetics Research Unit, Department of Biological and Environmental Sciences, University of Helsinki, PO Box 65, FI-00014 Helsinki, Finland.
Tel.: +358 9 191 57807; fax: +358 9 191 57694;
e-mail: abigel.gonda@helsinki.fi

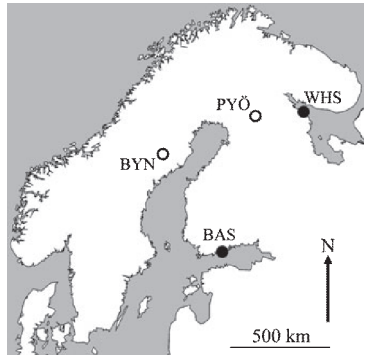


Fig. 1 Map showing the location of the study populations. Full circles denote marine populations: BAS, Baltic Sea population near Helsinki; WHS, White Sea population at Levin Navolok Bay; open circles denote ponds: PYÖ, Pyöreälampi (small isolated pond); BYN, Bynästjärnen (small isolated pond).

Materials and methods

Sampling, breeding and rearing

We collected adult nine-spined sticklebacks from four populations (Fig. 1) during May and June 2007. The two isolated ponds (surface area < 5 ha) were Pyöreälampi (Finland) and Bynästjärnen (Sweden) where the only representatives of other fish species were a few, recently introduced small-bodied whitefish (*Coregonus lavaretus*) in Pyöreälampi (information from the Oulanka Research Station). Our sample areas became free from ice around 8000 years ago after the last glaciation (e.g. Eronen *et al.*, 2001) so the colonization of these ponds must have happened after that. The marine samples came from the Baltic Sea (Helsinki, Finland) and White Sea (Levin Navolok Bay, Russia; Fig. 1). Marine nine-spined sticklebacks belong to a diverse fish community consisting of a large number of potential predators and competitors. In turn, in the ponds there are no predatory fish present, and the interspecific competition posed by other fish species is absent or negligible. Predation by aquatic insects and cannibalism at very early stages might be relevant in both habitats. The ponds exhibit very simple physical structure when compared to marine environments.

After collection, fish were moved to the aquaculture facilities of the University of Helsinki. Artificial crosses were made at the end of June. Five full-sib families were produced from every population *in vitro*. After hatching, each family was divided into two replicates and a maximum of 40 fish per replicate were placed in 10 L aerated plastic tanks. After 2–3 weeks, the fish were moved to similar tanks with mosquito nets at their sides that were placed into 140 L big plastic tanks (eight 10 L tanks in each) with an open, one-way water-flow. Family replicates were placed in different large tanks. After another 3–4 weeks, population pools were formed with families and replicates being equally represented. We aimed to get 100 offspring (= 5 families per population and 20 individuals per family) from each population [from the Baltic Sea, we could only get 93 (26, 24, 21, 16 and 6 per family) individuals]. Plastic tanks (140 L) were divided into halves by mosquito net and set with an open, one-way waterflow. Each population pool was divided into two replicates, and replicates were placed randomly into halves of the 140 L plastic tanks. Water temperature was set to 17 °C. Fish were fed live brine shrimp (*Artemia* sp.) nauplii first, and then frozen

crustaceans (*Cyclops* sp.) and bloodworms (Chironomidae sp.) *ad libitum*. We started with a 24 h light photoperiod, and changed it during 12 days gradually to a 12 h light/12 h dark photoperiod 12 weeks after hatching to avoid fish turning into reproductive condition. Better mimicking of the natural photoperiod change would have been very challenging to achieve due to the latitudinal differences in the source populations. We note that the number of replicate populations per habitat ($N = 2$) and independent families per population ($N = 5$) are limited, but considering that our populations are geographically (Fig. 1) and genetically (pairwise F_{ST}-s between 0.1 and 0.8; T. Shikano, G. Herczeg & J. Merilä, unpublished data) isolated, the data should be adequate for initial tests of the questions posed.

Brain measurements

Fish were overanaesthetized (with tricaine methanesulphonate) at the age of 5 months. A randomly chosen 15 individuals (seven and eight from the two replicates respectively) per population were measured. After measuring body weight to the nearest 0.01 g with a digital balance and standard length to the nearest 0.01 mm with digital callipers, brains of the fish were dissected and put into 4% formalin – 0.1 M phosphate-buffered saline solution. After 48 h fixation, photographs were made of the brains from dorsal, right lateral and ventral aspects with a digital camera (Canon EOS 10D; Canon Inc., Tokyo, Japan) connected to a dissecting microscope (Wild M5A; Wild, Heerbrugg, Switzerland). For bilateral structures, only the right parts were measured.

Width, height and length of the brain and five different brain parts – bulbus olfactorius, telencephalon, optic tectum, cerebellum and hypothalamus – were measured from the digital photographs using tpsDig 1.37 (Rohlf, 2002) software. They were defined as the greatest distance enclosed by the given structure. For a detailed description of the measurements see Pollen *et al.* (2007), whose measurement procedures we followed. We calculated the volume of the different brain parts according to the ellipsoid model (e.g. Huber *et al.*, 1997; Pollen *et al.*, 2007):

$$V = (L \times W \times H)\pi/6 \quad (1)$$

where V denotes the volume estimate, and L, W and H are the length, width and height of the given structure respectively. Although this model might not account for fine-scale variations in brain shape, it is a suitable measure for our purposes as we compared populations of the same species where drastic shape variations are not expected. The validity of the gross brain measures employed was tested by Pollen *et al.* (2007) and proved to provide consistent estimates of brain region volumes. For paired structures we used a doubled volume estimate of right side measurements. The volume of the total brain was estimated both with the equation suggested by Pollen *et al.* (2007):

$$V = (L \times W \times H)\pi/(6 \times 1.23) \quad (2)$$

and by summing the volumes of the different parts. The type of estimation did not alter the results qualitatively. Hence, only the results from the ellipsoid model are reported. Repeatability (R) of the volume estimates based on three independent measurements of 20 brains was high ($R > 0.86$, $P < 0.001$).

Analyses

To remove the allometric brain-body size effect (Northcutt *et al.*, 1978) all measures were $\log_{10}$ transformed. As the log standard length–log body weight relationship differed between the populations [General Linear Model (GLM), population × log standard length interaction: $F_{3,52} = 3.34$, $P = 0.03$], we corrected for both log standard length and weight in our subsequent analysis. First, a univariate GLM was used to test the difference in relative brain size between the populations. Log brain volume was defined as the dependent variable, population as the fixed factor and log body weight and log standard length as covariates. Pairwise *post-hoc* tests (LSD tests) were used in determining the significant population differences. Second, to test directly for habitat dependence, we used a General Linear Mixed Model (GLMM). Here, we entered log brain volume as dependent variable, habitat type (marine vs. pond) as fixed factor, population nested within habitat type as a random factor, and log body weight and log standard length as covariates.

We conducted a multivariate GLM to test for differences in brain structure with (log) brain parts as dependent variables, population as a fixed factor, and log body weight, log standard length and log brain volume as covariates. Upon significant multivariate effects, we ran univariate tests followed by LSD tests. To test directly for habitat specificity, we applied separate GLMMs on the variables found significant in the univariate tests (viz. telencephalon, bulbus olfactorius, cerebellum). Here, the given brain part was the dependent variable, habitat type the fixed factor, population nested within habitat type the random factor, whereas log body weight, log standard length and log brain volume were the covariates. All analyses were carried out with the SPSS 16.0 for Windows software package (SPSS Inc., Chicago, IL, USA).

Results

Independently of the body size effects (log body weight: $F_{1,54} = 38.47$, $P < 0.0001$; log standard length: $F_{1,54} = 22.11$, $P < 0.0001$) there were significant differences in relative brain size between the populations ($F_{3,54} = 12.62$, $P < 0.0001$; Fig. 2a). *Post-hoc* comparisons revealed

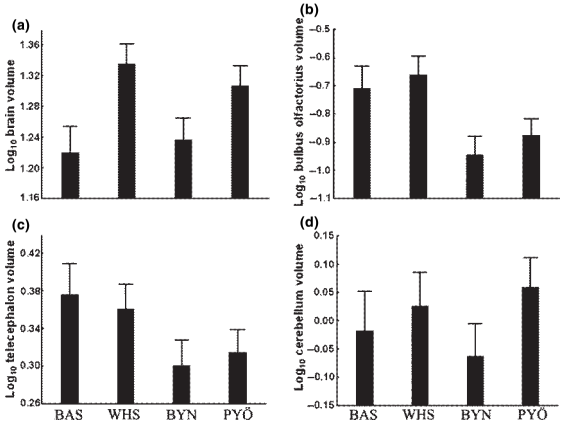


Fig. 2 Population differences in brain volume (corrected for body length and weight), and in the volume of different brain parts (corrected for body length, weight and brain volume). Least squares means + 95% confidence intervals are shown. BAS, Baltic Sea at Helsinki; WHS, White Sea at Levin Navolok Bay; PYÖ, Pyöreälampi (small isolated pond); BYN, Bynästjärnen (small isolated pond).

a habitat-independent pattern (Fig. 2a; White Sea > Baltic Sea; Pyöreälampi > Bynästjärnen; Pyöreälampi > Baltic Sea; White Sea > Bynästjärnen; all $P < 0.002$; all other pairs did not differ). Result of the GLMM analyses supported the results of GLM: after correcting for body size effects (log body weight: $F_{1,54.512} = 37.64$, $P < 0.0001$; log standard length: $F_{1,55.586} = 20.68$, $P < 0.0001$) there were no significant differences among the habitat types ($F_{1,2.085} = 0.01$, $P = 0.92$). Population within habitat type was also nonsignificant ($Z = 0.94$, $P = 0.35$).

After correcting for log weight (Wilks' $\lambda_{5,49} = 0.86$, $P = 0.18$), log standard length (Wilks' $\lambda_{5,49} = 0.91$, $P = 0.42$) and log brain volume (Wilks' $\lambda_{5,49} = 0.12$, $P < 0.0001$), our multivariate GLM revealed significant differences in sizes of different brain parts between the populations (Wilks' $\lambda_{15,135.669} = 0.39$, $P < 0.0001$). In the subsequent univariate analyses, we found significant population differences in three brain parts: bulbus olfactorius ($F_{3,53} = 12.05$, $P < 0.0001$; Fig. 2b), telencephalon ($F_{3,53} = 4.56$, $P = 0.006$; Fig. 2c) and cerebellum ($F_{3,53} = 4.00$, $P = 0.012$; Fig. 2d). Size of the optic tectum ($F_{3,53} = 1.76$, $P = 0.17$) and hypothalamus ($F_{3,53} = 1.10$, $P = 0.36$) did not differ significantly between populations. Pairwise *post-hoc* comparisons revealed a systematic difference between fish from marine and pond environments; marine fish having larger bulbus olfactorius (between habitat types: all $P < 0.008$; within habitat types: all $P > 0.1$) and telencephalon (between habitat types: all $P < 0.017$; within habitat types: all $P > 0.42$). However, the pattern in cerebellum differences was less clear, only the two small pond populations being significantly different from each other ($P = 0.002$; all other $P > 0.08$). Results of GLMM analyses supported the results of the GLM analyses. Habitat-specific differences were found in case of telencephalon ($F_{1.5,565} = 10.18$, $P = 0.021$) after correcting for size effects (log body weight: $F_{1,25.517} = 0.04$, $P = 0.85$; log standard length: $F_{1,11.507} = 0.13$, $P = 0.72$; log brain volume: $F_{1,25.892} = 115.18$, $P < 0.0001$), whereas populations did not differ within habitat types ($Z = 0.33$, $P = 0.74$). Also, we found habitat-specific differences in the case of bulbus olfactorius ($F_{1,3.776} = 28.69$, $P = 0.007$) after correcting for size effects (log body weight: $F_{1,18.135} = 0.001$, $P = 0.97$; log standard length: $F_{1,4.412} = 3.44$, $P = 0.10$; log brain volume: $F_{1,19.554} = 3.16$, $P = 0.09$), whereas populations did not differ within habitat types ($Z = 0.21$, $P = 0.83$). In the case of the cerebellum, we did not find habitat-specific differences ($F_{1.2,362} = 0.005$, $P = 0.95$), after correcting for size effects (log body weight: $F_{1,49.137} = 3.95$, $P = 0.05$; log standard length: $F_{1,35.594} = 2.85$, $P = 0.10$; log brain volume: $F_{1,44.853} = 22.40$, $P < 0.0001$), whereas populations did not differ within habitat types ($Z = 0.79$, $P = 0.43$).

Discussion

Our results indicate that different populations of the same species can differ genetically in their relative brain size, and in the relative sizes of different brain parts. In particular, the observed divergence in the size of bulbus olfactorius and telencephalon was explained by habitat type (marine vs. pond) rather than by population origin. This is the first data to suggest habitat-dependent genetically based intraspecific divergence in brain architecture among natural populations. This habitat-specific

nature of the differences suggests that natural selection is the agent behind the observed divergence. As our study area was covered with ice sheets until about ca. 8000 years ago (Eronen *et al.*, 2001), the results also indicate that observed differences have evolved relatively rapidly.

Independent evolution of the same phenotype in natural populations occupying similar habitats strongly implies natural selection as the causal agent (e.g. Clarke, 1975; Endler, 1986; Schluter & Nagel, 1995; Foster, 1999; McGuigan *et al.*, 2005). The repeated, habitat-specific differences in the relative size of two main brain regions, bulbus olfactorius and telencephalon, strongly suggest adaptive basis of observed differentiation. As the brain morphology differed between the habitat types systematically with marine populations having larger bulbus olfactorius and telencephalon than fish from ponds, we hypothesize that the (i) lack of interactions with other fish species (competitors and/or predators) and/or (ii) reduced environmental complexity in pond environments have relaxed selection on neural processes supported by bulbus olfactorius and telencephalon and resulted in their reduction – a pattern easily understandable in the light of the extremely high development and maintenance costs of brain tissue (Aiello & Wheele, 1995). Similar adaptive reductions in brain parts have been demonstrated in interspecific comparisons (Niven, 2005).

Previous interspecific studies on fish have found positive correlation between telencephalon size and habitat complexity, as well as between bulbus olfactorius size and water turbidity (Huber *et al.*, 1997). Telencephalon has also been found to be larger in polygamous than in monogamous species (Pollen *et al.*, 2007). Telencephalon-ablated fish exhibit significantly diminished rates of learning and habituation (e.g. Laming & McKinley, 1990) and display a deficit in avoidance learning (Portavella *et al.*, 2003). In-depth anatomical analyses indicate that the telencephalon is involved in spatial and emotional learning (for a review, see e.g. Broglio *et al.*, 2003). Even considering that correlations do not allow us to draw firm conclusions about causation, our results together with inference from previous studies suggest that the complexity of biotic environment (e.g. number of fish species), habitat complexity and the importance of learning abilities may be key factors in explaining telencephalon size differences between our nine-spined stickleback populations. The bulbus olfactorius differences may relate to lower visibility in the marine environment, and/or reduced utility of chemosensory functions in the isolated ponds lacking predatory fish.

Significant cerebellum size differences between the pond populations are also intriguing. The cerebellum appears to play a role in spatial orientation, motor coordination and eye movement (Kotrschal *et al.*, 1998), and correlates positively with species diversity and habitat complexity in cichlid fishes (Pollen *et al.*, 2007). In our case, the observed pattern cannot be explained with habitat differences, and thus, requires further investigations.

We also found that mean relative brain volume differed between the study populations, but not in a habitat-specific fashion. In theory, the population pairs with larger (Pyöreälampi and White Sea) and smaller (Bynästjärnen and Baltic Sea) relative brain volumes might share common ancestors (they indeed belong to different drainages) that were genetically differentiated in brain size already before the current populations were formed. Yet, it is also true that we have not measured or identified all possible environmental and ecological differences that might exist between different populations. The fact that fish from different populations, born and reared under standardized laboratory conditions in the absence of any energetic constraints, develop brains of different size suggest that there are some yet unidentified selection pressures which underlie these differences. Identification of these differences remains a challenge for future studies.

In summary, we found genetically based differences in relative brain size and brain architecture among wild nine-spined stickleback populations occupying contrasting habitats. The systematic differences in the relative volume of bulbus olfactorius and telencephalon between habitat types suggest that the divergence is caused by habitat-specific natural selection. This inference is supported by the fact that the direction of differences accords with expectations based on what is known about the function of these brain structures. However, further studies with more extensive replication, as well as confirmatory studies with other species, are needed to establish the generality of the findings.

Acknowledgments

We thank Victor Berger, Göran Englund, Tuomas Leinonen, Daniel Lussetti and Pirkko Siikamäki for helping us in organizing and executing the field sampling. Special thanks to the Oulanka Research Station and White Sea Biological Station for their help and support. We are highly indebted to Hans Hofmann and Pertti Panula for helping with practical issues related to the preparation and measurement of brains. We thank László Garamszegi for his constructive comments leading to improvements of the manuscript and John Loehr for correcting the English. The study was supported by the Academy of Finland and the Centre for International Mobility. The experiments were performed under the license of the Helsinki University Animal Experimentation Committee.

References

Aiello, L.C. & Wheele, P. 1995. The expensive tissue hypothesis – the brain and digestive system in human and primate evolution. *Curr. Anthropol.* **36**: 199–221.

Bănărescu, P.M. & Paepke, H.J. 2001. *The Freshwater Fishes of Europe*, Vol. 5/III. AULA-Verlag, Wiebelsheim.
Broglio, C., Rodriguez, F. & Salas, C. 2003. Spatial cognition and its neural basis in teleost fish. *Fish Fish.* **4**: 247–255.
Burns, J.G. & Rodd, H. 2008. Hastiness, brain size and predation regime affect the performance of wild guppies in a spatial memory task. *Anim. Behav.* **76**: 911–922.
Clarke, B.C. 1975. The contribution of ecological genetics to evolutionary theory: detecting the direct effects of natural selection on particularly polymorphic loci. *Genetics* **79**(Suppl.): 101–113.
Clutton-Brock, T.H. & Harvey, P.H. 1980. Primates, brains and ecology. *J. Zool.* **190**: 309–323.
Day, L.B., Westcott, D.A. & Olster, D.H. 2005. Evolution of bower complexity and cerebellum size in bowerbirds. *Brain Behav. Evol.* **66**: 62–72.
Drake, J.M. 2007. Parental investment and fecundity, but not brain size, are associated with establishment success in introduced fishes. *Funct. Ecol.* **21**: 963–968.
Endler, J.A. 1986. *Natural Selection in the Wild*. Princeton University Press, Princeton.
Eronen, M., Gluckert, G., Hatakka, L., van der Plassche, O., van der Plicht, J. & Rantala, P. 2001. Rates of Holocene isostatic uplift and relative sea-level lowering of the Baltic in SW Finland based on studies of isolation contacts. *Boreas* **30**: 17–30.
Foster, S.A. 1999. The geography of behaviour: an evolutionary perspective. *Trends Ecol. Evol.* **14**: 190–195.
Garamszegi, L.Z. & Eens, M. 2004a. The evolution of hippocampus volume and brain size in relation to food hoarding in birds. *Ecol. Lett.* **7**: 1216–1224.
Garamszegi, L.Z. & Eens, M. 2004b. Brain space for a learned task: strong intraspecific evidence for neural correlates of singing behavior in songbirds. *Brain. Res. Revs.* **44**: 187–193.
Gonzalez-Voyer, A., Winberg, S. & Kolm, N. 2009. Social fishes and single mothers: brain evolution in African cichlids. *Proc. R. Soc. B* **276**: 161–167.
Harvey, P.H., Clutton-Brock, T.H. & Mace, G.M. 1980. Brain size and ecology in small mammals and primates. *Proc. Natl. Acad. Sci. USA* **77**: 4387–4389.
Healy, S.D. & Rowe, C. 2007. A critique of comparative studies of brain size. *Proc. R. Soc. B* **274**: 453–464.
Huber, R., Staaden, M.J., van Kaufman, L.S. & Liem, K.F. 1997. Microhabitat use, trophic patterns, and the evolution of brain structure in African cichlids. *Brain Behav. Evol.* **50**: 167–182.
Ishikawa, Y., Yoshimoto, M., Yamamoto, N. & Ito, H. 1999. Different brain morphologies from different genotyoes in a single teleost species, the medaka (*Oryzias latipes*). *Brain Behav. Evol.* **53**: 2–9.
Karlen, S.J. & Krubitzer, L. 2006. Phenotypic diversity is the cornerstone of evolution: variation in cortical field size within short tailed opossums. *J. Comp. Neurol.* **499**: 990–999.
Kaufman, J.A. 2003. On the Expensive Tissue Hypothesis: independent support from highly encephalized fish. *Curr. Anthropol.* **44**: 705–706.
Kihslinger, R.L. & Nevitt, G.A. 2006. Early rearing environment impacts cerebellar growth in juvenile salmon. *J. Exp. Biol.* **209**: 504–509.
Kotrschal, K., Van Staaden, M.J. & Huber, R. 1998. Fish brains: evolution and environmental relationships. *Rev. Fish Biol. Fish.* **8**: 373–408.
Laming, P.R. & McKinley, S.J. 1990. Habituation in goldfish (*Carassius auratus*) is impaired by increased interstimulus interval, interval variability, and telencephalic ablation. *Behav. Neurosci.* **104**: 869–875.
Madden, J. 2001. Sex, bowers and brains. *Proc. R. Soc Lond. B* **268**: 833–838.
Maguire, E.A., Gadian, D.G., Johnsrude, I.S., Good, C.D., Ashburner, J., Frackowiak, R.S.J. & Frith, C.D. 2000. Navigation-related structural change in the hippocampi of taxi drivers. *Proc. Natl. Acad. Sci. USA* **97**: 4397–4403.
McGuigan, K., Chenoweth, S.F. & Blows, M.W. 2005. Phenotypic divergence along lines of genetic variance. *Am. Nat.* **165**: 32–43.
Merilä, J. & Crnokrak, P. 2001. Comparison of genetic differentiation at marker loci and quantitative traits. *J. Evol. Biol.* **14**: 892–903.
Niven, J.E. 2005. Brain evolution: getting better all the time? *Curr. Biol.* **15**: R624–R626.
Northcutt, R.G., Neary, T.J. & Senn, D.G. 1978. Observations on the brain of coelacanth *Latimeria chalumnae*: external anatomy and quantitative analysis. *J. Morphol.* **155**: 181–192.
Pitnick, S., Jones, K.E. & Wilkinson, G.S. 2006. Mating system and brain size in bats. *Proc. R. Soc. B* **273**: 719–724.
Pollen, A.A., Dobberfuhl, A.P., Scace, J., Igulu, M.M., Renn, S.C.P., Shumway, C.A. & Hofmann, H.A. 2007. Environmental complexity and social organization sculpt the brain in Lake Tanganyikan cichlid fish. *Brain Behav. Evol.* **70**: 21–39.
Portavella, M., Salas, C., Vargas, J.P. & Papini, M.R. 2003. Involvement of telencephalon in spaced-trial avoidance learning in the goldfish (*Carassius auratus*). *Phys. Behav.* **80**: 49–56.
Rohlf, F.J. 2002. *tpsDig, Digitize Landmarks and Outlines, Version 1.37*. Department of Ecology and Evolution, State University of New York at Stony Brook.
Schluter, D. & Nagel, L.M. 1995. Parallel speciation by natural selection. *Am. Nat.* **146**: 292–301.
Sol, D., Duncan, R.P., Blackburn, T.M., Cassey, P. & Lefebre, L. 2005. Big brain, enhanced cognition, and response of bird to novel environments. *Proc. Natl. Acad. Sci. U.S.A.* **102**: 5460–6465.
Sol, D., Bacher, S., Reader, S.M. & Lefebvre, L. 2008. Brain size predicts the success of mammal species introduced into novel environments. *Am. Nat.* **172**: S63–S71.
Tramontin, A.D. & Brenowitz, E.A. 2000. Seasonal plasticity in adult brain. *Trends Neurosci.* **23**: 251–258.

Received 8 October 2008; accepted 8 May 2009

PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2009) **276**, 2085–2092
doi:10.1098/rspb.2009.0026
Published online 11 March 2009

Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain

Abigél Gonda*, Gábor Herczeg and Juha Merilä

Ecological Genetic Research Unit, Department of Biological and Environmental Sciences, University of Helsinki, PO Box 65, 00014 Helsinki, Finland

The influence of environmental complexity on brain development has been demonstrated in a number of taxa, but the potential influence of social environment on neural architecture remains largely unexplored. We investigated experimentally the influence of social environment on the development of different brain parts in geographically and genetically isolated and ecologically divergent populations of nine-spined sticklebacks (*Pungitius pungitius*). Fish from two marine and two pond populations were reared in the laboratory from eggs to adulthood either individually or in groups. Group-reared pond fish developed relatively smaller brains than those reared individually, but no such difference was found in marine fish. Group-reared fish from both pond and marine populations developed larger tecta optica and smaller bulbi olfactorii than individually reared fish. The fact that the social environment effect on brain size differed between marine and pond origin fish is in agreement with the previous research, showing that pond fish pay a high developmental cost from grouping while marine fish do not. Our results demonstrate that social environment has strong effects on the development of the stickleback brain, and on the brain's sensory neural centres in particular. The potential adaptive significance of the observed brain-size plasticity is discussed.

Keywords: brain size; brain architecture; group living; nine-spined stickleback; phenotypic plasticity; *Pungitius*

1. INTRODUCTION

Several forms of plasticity in brain architecture have been demonstrated at different neuroanatomical levels and life stages in numerous taxa, including mammals, birds and fishes (e.g. Diamond *et al.* 1966; Rosenzweig & Bennett 1969; Kempermann *et al.* 1997; Tramontin & Brenowitz 2000; Zupanc 2001; Draganski & May 2008). During the past few decades, experimental studies have shed light on the effects of abiotic and biotic environmental complexities on the development of neural architecture (reviewed in Van Praag *et al.* 2000; Mohammed *et al.* 2002). For instance, rodents kept in stimulus-rich environments increased their brain size (Diamond *et al.* 1966; Rosenzweig & Bennett 1969), had more hippocampal neurons (Kempermann *et al.* 1997) and showed an elevated level of neurogenesis (Kempermann *et al.* 1997; Nilsson *et al.* 1999) as compared with those kept in a stimulus-poor environment. Chinook salmon (*Oncorhynchus tshawytscha*) reared in overly simplified hatchery conditions developed smaller bulbi olfactorii and telencephala as compared with wild conspecifics (Kihslinger *et al.* 2006). Kihslinger & Nevitt (2006) demonstrated that simply adding a few rocks in the rearing tanks resulted in increased cerebellum size in salmon (*Oncorhynchus mykiss*) alevins, while structural complexity of the abiotic environment affected the rate of cell proliferation in the telencephalon of juvenile coho salmon (*Oncorhynchus kisutch*; Lema *et al.* 2005). Structurally enriched environment has also been shown to increase foraging skills and learning ability in Atlantic salmon (*Salmo salar*; Brown *et al.* 2003). Obviously, brain architecture and behaviour are expected to be correlated both within and between species. In line with that expectation, Burns & Rodd (2008) have demonstrated a negative correlation between 'hastiness' and telencephalon size in guppies (*Poecilia reticulata*). On a larger scale, several comparative studies have revealed that behavioural and neural complexity appears to evolve in concert (e.g. Lefebvre *et al.* 1997; Reader & Laland 2002; Gonzalez-Voyer *et al.* 2009).

Social environment is also implicated as an important factor in shaping the ontogeny and evolution of brain architecture. For instance, the large brains of primates are thought to be a consequence of living in complex societies (Dunbar & Shultz 2007). Coevolution of sociality and brain size has also been demonstrated in other mammals (Perez-Barberia *et al.* 2007), while recent studies have started to uncover the importance of parental care-type and pair-bonding in the brain-size evolution of fishes (Pollen *et al.* 2007; Gonzalez-Voyer *et al.* 2009). However, apart from these interspecific comparative studies, only very few experimental studies have investigated the effects of sociality on brain architecture. Social isolation was found to decrease the number of new neurons in the dentate gyrus of prairie voles (*Microtus ochrogaster*; Fowler *et al.* 2002), while greater social complexity increased neuronal recruitment in birds (Lipkind *et al.* 2002; Adar *et al.* 2008).

Adult neurogenesis is limited to a few areas of the brain in mammals (Gould *et al.* 1999; Hastings *et al.* 2000, 2001), yet several studies have demonstrated its more widespread occurrence in birds (e.g. Reh & Fischer 2001). In comparison with mammals and birds, neurogenesis persists longer into adulthood in reptiles (Font *et al.* 2001) and fishes (Zupanc & Horschke 1995; Zupanc 2001, 2006), contributing to lifelong growth of brain size and thereby the potential for plastic responses to environmental heterogeneity (Birse *et al.* 1980; Raymond & Easter 1983; Zupanc & Horschke 1995). Hence, fishes provide an excellent model for neural plasticity studies. The effect of abiotic environment on the development of brain architecture in salmonid fishes has been demonstrated (e.g. Kihslinger & Nevitt 2006; Kihslinger *et al.* 2006). However, despite the widespread occurrence of group living in numerous fish taxa (e.g. Pitcher & Parrish 1993; Krause & Ruxton 2002), no studies of the potential effects of social environment on brain architecture in fishes have been conducted. Likewise, studies investigating the possibility that genetically based population-level differences in brain development are due to sociality are as yet to be conducted. Such differences could be expected to occur if the costs and benefits of grouping differ among populations residing in different selective environments.

The aim of this study was to investigate the long-term effects of social environment on brain development of nine-spined sticklebacks (*Pungitius pungitius*), and to compare the effects between populations originating from contrasting environments. This was done by comparing the relative size of brains and five different brain regions of adult fish subjected to different social environment treatments in the laboratory from hatching until adulthood. We were interested in addressing the following questions. (i) Is there any difference in relative brain size of nine-spined sticklebacks reared either individually or in groups? (ii) Which parts of the brain are the most affected by these conditions? (iii) Are there any population or habitat-specific differences in the detected patterns? The latter could be expected because the study populations originated from two contrasting environments (*viz.* marine and pond environments) where the costs and benefits of grouping are different. Pond fish grow faster (G. Herczeg, A. Gonda & J. Merilä, unpublished data), are more aggressive, are bolder, have higher drive to feed (Herczeg *et al.* 2009*a*), and probably, as a consequence, display a higher cost of grouping (Herczeg *et al.* 2009*b*) than their marine conspecifics. Hence, we could formulate two main hypotheses. First, we hypothesize that there should be a habitat-specific treatment effect on relative brain size due to the habitat-specific differences in the costs of grouping (i.e. pond fish should have smaller brains when reared in groups than when reared individually). Second, we hypothesize about habitat-independent treatment effects on brain parts involved in communication or, more generally, in perception of the social environment. Here, we expected that the tectum opticum (the visual centre) will be enlarged in group-reared fish as compared with individual-reared conspecifics.

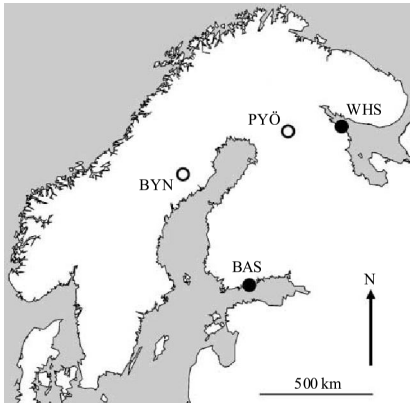


Figure 1. Map showing the location of the study populations. BAS, Baltic Sea, Finland; WHS, White Sea, Russia; PYÖ, Pyöreälampi, Finland; BYN, Bynästjärnen, Sweden. Open circles, small isolated ponds; filled circles, marine populations.

2. MATERIAL AND METHODS

(a) *Field sampling and study populations*

Adult nine-spined sticklebacks were collected from late May to early June of 2007, immediately before the peak of the breeding season, with the aid of minnow traps and seine nets from four populations representing two contrasting habitat types. Although it is known that sampling can introduce a bias towards bolder-than-average fish (Biro & Dingemanse 2009), this effect is hard to avoid, and we believe that the role of this possible bias is negligible in our case. These were two marine populations from the Baltic Sea near Helsinki (Finland) and the White Sea in Levin Navolok Bay (Russia), and two pond populations from Bynästjärnen (Sweden) and Pyöreälampi (Finland; figure 1). The marine sampling sites were shallow coastal bays close to creek inlets (Baltic Sea being a brackish water environment), representing low-salinity sea habitats. Even though we could sample only two replicate populations per habitat type, the large geographical (above 500 km) and genetic (based on highly polymorphic microsatellite markers; T. Shikano, G. Herczeg & J. Merilä, unpublished data) distance made them truly independent. The surface area of ponds was less than 5 ha and their maximum depth around 10 m. The habitats differ in several respects: nine-spined stickleback is the only fish species in the ponds apart from a small number of recently introduced small-bodied whitefish (*Coregonus lavaretus*) in Pyöreälampi. Based on diet analyses (e.g. Kahilainen *et al.* 2004), these whitefish are a potential competitor but not a predator of the nine-spined sticklebacks. It is noteworthy that we never caught a single whitefish among the thousands of sticklebacks during our extensive sampling in Pyöreälampi. Thus, pond sticklebacks experience no fish predation and no (or negligible) interspecific competition. By contrast, marine sticklebacks face several types of predatory fishes and interspecific competitors. These differences have resulted in entirely different evolutionary constraints of group living in the populations used here: pond fish suffer from reduced growth when kept in groups while marine fish do not (Herczeg *et al.* 2009*b*).

(b) *Breeding conditions and experimental design*

After collection, adult fish were transported to the aquaculture facilities of the University of Helsinki and kept at 17°C under permanent light and fed with frozen bloodworms

* Author for correspondence (abigel.gonda@helsinki.fi).

Received 6 January 2009
Accepted 10 February 2009

(Chironomidae sp.) until a sufficient number of fish had attained reproductive condition. Both wild-caught adults and all their offspring (see below) were kept and raised in freshwater. Five artificial crosses per population were made in the last week of June. The clutches were placed into 1.4 l tanks of two Allentown Zebrafish Rack Systems (hereafter 'rack'; Aquaneering Inc., San Diego, CA, USA). Racks had closed water circulating systems with multi-level filtering (physical, chemical, biological and UV filters) and inbuilt thermostats. Unfertilized eggs were removed daily. After hatching, 10 fish per family (i.e. 50 fish per population) were placed individually and randomly into the 1.4 l tanks of the two racks (hereafter individual treatment). The transparent plastic tanks were separated from each other with white panels to block visual contact between neighbours. Chemical contact could not be blocked due to the closed water system.

Of the remaining fish, in the second treatment (hereafter group treatment), a maximum of 80 individuals (depending on the size of the family) per family were divided into two replicates and placed in well-aerated 10 l plastic tanks. After three to four weeks, fish were transported to similar 10 l tanks with mosquito nets at the sides, and these tanks were placed in larger plastic tanks (76×54×40 cm, length, width and height, respectively; eight 10 l tanks in each) set with an open, one-way water flow. The 10 l tanks were placed randomly into the large tanks, and replicates within family were placed into different large tanks. After another three to four weeks (depending on the day of fertilization) 20 fish per family were chosen (replicates equally represented) and pooled within populations, resulting in pools of 100 fish per population. From the Baltic population, equal family representation and reaching $n=100$ were impossible because of the low number of individuals in the original families and the subsequent mortality. Here, 93 fish were pooled (26, 24, 21, 16 and 6 per family, respectively). Each new population pool was divided into two replicates. The replicates were placed randomly into halves of the larger (76×54×40 cm) plastic tanks halved by mosquito net and set with an open, one-way water flow. The replicates within populations were placed into different tanks. The water volume was set to 140 l in the larger tanks; hence, the *per capita* water volume (1.4 l), or in other words the fish density, was similar between treatments from this point onwards. In short, only chemosensory clues of conspecifics were present in the individual treatment, while visual, chemosensory and tactile cues were all present in the group treatment.

In both treatments, the temperature was set to 17°C throughout the experiment. We changed from a 24-hour light (natural at high latitudes in summer) cycle to a 12 L : 12 D periodism gradually during the course of one week after week 12. Owing to the latitudinal differences between the populations (figure 1), we did not attempt to mimic the natural light regimes any more closely. Fish were *ad libitum* fed two times per day. Feeding was started with live brine shrimp (*Artemia salina*); as the fish grew, we switched to frozen copepeods (*Cyclops* sp.) and then to frozen bloodworms. No gravel or other physical structures were presented in the rearing environments.

(c) *Brain measurements*

At the age of five months, when fish reached adult size (standard length from the tip of the nose to the tail base=4–7 cm; e.g. Bănărescu & Paepke 2001), 15 individuals from every population and every treatment were killed by an overdose of MS 222 (tricaine methanesulphonate). Individuals from the individual treatments represented families equally, and individuals from the group treatment were selected randomly from the mixed population pools (replicates represented evenly). After over-anaesthetizing the fish, their body weight was measured to the nearest 0.01 g with a digital balance and their standard length to the nearest 0.01 mm with a digital calliper. Then the brains of the fish were dissected and put into a 4 per cent formalin–0.1 M phosphate-buffered saline solution for 48 hours of fixation. After that, digital photographs were taken of the brains from three viewpoints (dorsal, right lateral and ventral) with a digital camera (Canon EOS 10D, Canon Inc., Tokyo, Japan) through a connected dissecting microscope (Wild M5A, Heerbrugg, Switzerland). A scale was positioned in each photograph for later measurements. Brains were positioned symmetrically and in a horizontal position by eye. We estimated the repeatability of our measurements based on three repeated independent measures of a subsample of brains ($n=20$) and found that all measurements (see below) were highly repeatable (all $r>0.8$).

The size of the brain and five different brain parts—bulbus olfactorius, telencephalon, tectum opticum, cerebellum and hypothalamus—were measured from the digital photographs with TPSDIG v. 1.37 software (Rohlf 2002). The width, height and length of each structure were taken and defined as the greatest distance enclosed by the given structure. The measures were perpendicular to the midline in the case of width, parallel to the projection of the brain in the case of length and perpendicular to the projection of the brain in the case of height. A detailed description of the measurements is given by Pollen *et al.* (2007), whose measurement procedures we followed. The volume of the total brain and the different brain parts was estimated according to the ellipsoid model (e.g. Huber *et al.* 1997; Pollen *et al.* 2007). This model might not account for fine-scale changes in brain shape, but it should be suitable for the purpose of our study as we compared populations of the same species where large shape changes are not expected. These estimates were validated by Pollen *et al.* (2007), who found that they provided consistent volume estimates of different brain regions. The volume (V) of the different brain parts was calculated as

$$V=(L\times W\times H)\pi/6, \tag{2.1}$$

where L, W and H denote the length, width and height of the given structure, respectively. For paired structures we used a doubled volume estimate of right side measurements. The total volume of the brain was estimated in two different ways. First, we used the equation

$$V=(L\times W\times H)\pi/(6\times 1.23) \tag{2.2}$$

(Pollen *et al.* 2007); and, second, we simply summed the volumes of the different parts. The method of calculation did not influence the results qualitatively. Hence, only the results from the ellipsoid model are reported. We note that this method did not allow us to analyse fine-scale structural differences within brain parts, but significant differences at measures used would indeed indicate large treatment and/or population effects.

(d) *Data analyses*

All morphological variables were log transformed to correct for the allometric relationship between brain size and body size (Northcutt *et al.* 1978) and to achieve a linear relationship between them. The transformed values were used in all analyses. A general linear mixed model (GLMM) was used to test for the habitat and treatment effects on brain size. Because we found a marginally significant difference in the body weight–standard length relationship between the populations (GLM ANCOVA: $F_{3,112}=2.38$, $p=0.073$), we corrected for both body weight and standard length in our analyses. In the GLMM, brain volume was the dependent variable, treatment and habitat fixed factors, body weight and standard length covariates, and population nested in habitat type a random factor.

Since the different parts of the brain were not independent, a multivariate GLM was conducted to test for the treatment effects at the population level. In this analysis (MANCOVA), the size of bulbus olfactorius, telencephalon, tectum opticum, cerebellum and hypothalamus were defined as dependent variables, treatment and population as fixed factors and body weight, standard length and brain volume as covariates.

In all models, we included the interaction between the fixed factors. Analyses were carried out with the SPSS v. 16.0 for Windows (SPSS Inc., Chicago, IL) software package.

3. RESULTS

After correcting for size effects (body weight: $F_{1,112.55}=49.991$, $p<0.0001$; standard length: $F_{1,113.09}=34.866$, $p<0.0001$), a habitat-specific treatment effect on brain size was found (habitat×treatment interaction: $F_{1,112.16}=7.816$, $p=0.006$; figure 2). The main effects of treatment ($F_{1,112.34}=2.035$, $p=0.157$) and habitat ($F_{1,2.07}=0.146$, $p=0.738$) were insignificant, as was the effect of population within habitat type ($Z=0.978$, $p=0.328$). Pond fish grew smaller brains in the group treatment than in the individual treatment, while no such effect was observed in marine fish (figure 2).

After correcting for size effects (body weight: Wilks's $\lambda_{5,105}=0.922$, $p=0.124$; standard length: Wilks's $\lambda_{5,105}=0.921$, $p=0.117$; brain volume Wilks's $\lambda_{5,105}=0.145$, $p<0.0001$), multivariate GLM revealed a significant treatment (Wilks's $\lambda_{5,105}=0.828$, $p=0.001$) and population effects (Wilks's $\lambda_{15,290}=0.39$, $p<0.0001$) on different brain parts. The treatment×population interaction was insignificant (Wilks's $\lambda_{15,290}=0.92$, $p=0.86$). Univariate analyses of the data revealed significant treatment effect on two brain parts, the bulbus olfactorius ($F_{1,109}=11.22$, $p=0.001$; figure 3) and the tectum opticum ($F_{1,109}=7.72$, $p=0.006$; figure 3). The bulbus olfactorius was significantly larger, while the tectum opticum was smaller in fish from the individual treatment than in fish from the group treatment. The treatments did not affect the size of telencephalon ($F_{1,109}=0.16$, $p=0.69$), cerebellum ($F_{1,109}=2.52$, $p=0.115$) or hypothalamus ($F_{1,109}=0.288$, $p=0.593$). The treatment-independent population differences are not in the focus of the present paper and will be discussed elsewhere.

4. DISCUSSION

Our results demonstrate that social environment can have marked effects on the development of the nine-spined stickleback's brain. Fish originating from pond populations developed smaller brains when reared in groups than when reared alone, while fish originating from marine

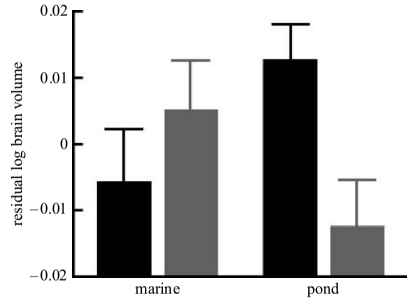


Figure 2. Social environmental effect on brain volume (mean+s.e.). Significant habitat-dependent treatment effect was found. Black bars, individual treatment; grey bars, group treatment.

populations showed a (insignificant) trend towards the opposite. According to our knowledge, this is the first time an interpopulation difference in brain-size plasticity during ontogenesis has been demonstrated. The fact that the difference in the level of plasticity was habitat- and not population-specific suggests that habitat-specific natural selection is the likely cause of the observed difference (cf. Clarke 1975; Endler 1986; McGuigan *et al.* 2005). We further discovered that social environment affected the development of different brain regions (*viz.* bulbus olfactorius and tectum opticum) in a similar manner in all populations. Individually reared fish receiving information from their conspecifics only via chemical cues developed significantly larger bulbi olfactorii than fish grown in groups. By contrast, group-reared fish subject to visual, chemical and tactile sensory inputs from conspecifics grew significantly larger tecta optica than individually reared fish. Size of telencephalon, cerebellum and hypothalamus appeared to be unaffected by social environment.

Population differences in learning and memorizing abilities (e.g. Mackney & Hughes 1995; Nelson *et al.* 1995; Girvan & Braithwaite 1998; Brown & Braithwaite 2005) and in brain architecture have been demonstrated in some taxa (Garamszegi & Eens 2004; Pravosudov *et al.* 2006; Brown *et al.* 2007; Burns & Rodd 2008; A. Gonda, G. Herczeg & J. Merilä, unpublished data). However, we are not aware of any study that would have investigated interpopulation variation in neural plasticity. In the present study, population differences in plasticity in response to social environment occurred between populations from two markedly different habitats in which the cost of sociality is expected and known to differ (Herczeg *et al.* 2009*a*,*b*).

Because marine nine-spined sticklebacks are under heavy fish predation throughout their lifespan, grouping can be beneficial in reducing predator-caused mortality and, assuming that food is patchier in marine than in pond environments, in increasing foraging efficiency too (Pitcher & Parrish 1993; Krause & Ruxton 2002). By contrast, intraspecific competition is expected to be one of the main biotic selective forces in pond sticklebacks. In fact, fish from ponds are more aggressive, bolder and have higher drive to feed (Herczeg *et al.* 2009*a*) than their

Downloaded from rspb.royalsocietypublishing.org on 12 November 2009

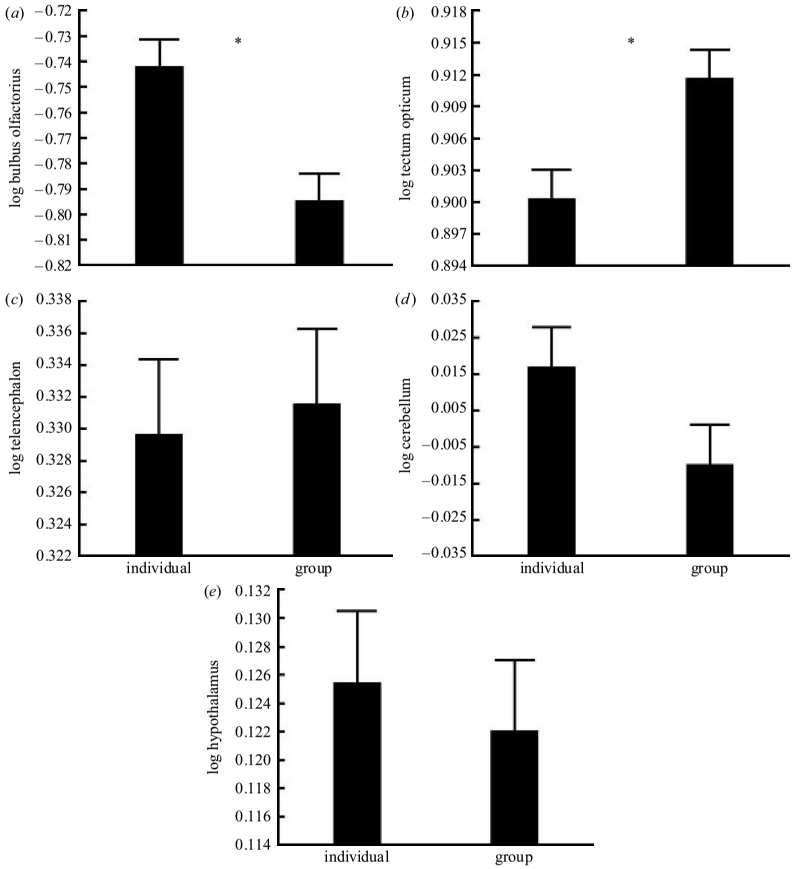


Figure 3. (*a–e*) Least-squares mean (+s.e.) size of brain parts in different social environmental treatments in different populations. Significant treatment effects are marked with asterisks.

marine conspecifics. Furthermore, and probably as a consequence, pond fish face high costs of grouping in terms of growth even when constraints originating from food limitation, predation, parasitism or reproduction are ruled out (Herczeg *et al.* 2009*b*). This happens irrespective of the fact that pond fish occur in high densities (G. Herczeg & A. Gonda, personal observation) and that, under stress, both pond and marine fish tend to group (Herczeg *et al.* 2009*b*). Therefore, group living or permanent contact with conspecifics can be considered to better reflect the natural situation for both marine and pond fish than living in isolation (which is hard to imagine in the studied habitat types), but also more stressful for pond than for marine fish. Considering that the brain is the most expensive tissue to develop and maintain (e.g. Aiello & Wheele 1995), the results showing that pond fish had smaller relative brain size when kept in groups than when kept alone, while marine fish showed some tendency towards opposite patterns, are not unexpected. However, it is interesting that the cost of grouping could manifest as a reduction in brain size in a situation where—due to the increased need during social interactions—one could actually expect larger brains to be developed. The fact that we used laboratory-reared fish suggests that the among-population patterns are likely to have a genetic basis, even though the possibility of maternal effects cannot be ruled out in our design. Furthermore, we found repeated, habitat-specific, population-independent differences that strongly support the role of natural selection in shaping the pattern (e.g. Clarke 1975; Endler 1986; Schluter & Nagel 1995; Foster 1999; McGuigan *et al.* 2005). We suggest that selection did not act directly on brain plasticity, but rather on the causes behind the differences in grouping costs between habitat types (e.g. behaviour), manifested as energetic constraints on brain development.

Previous studies in brain development have demonstrated that those parts of the brain that are likely to be important in a particular context develop more than those of less importance (Kihslinger & Nevitt 2006; Kihslinger *et al.* 2006; Lisney *et al.* 2007). It has also been shown that changes in demand alter the number and size of component elements, making the relative size of different brain parts a reliable predictor of their importance for the organism in question (Kotrschal *et al.* 1998). In our experiment, individually reared fish could only get information from their conspecifics by chemical cues, while visual, chemical and tactile cues were all available for group-reared fish. Our treatments were extremely simple in terms of abiotic complexity (we applied empty plastic tanks). Hence, one could expect that olfactory centres will be enlarged in the individual treatment, while visual centres will be enlarged in the group treatment. Our results are in line with these expectations: individually reared fish had larger bulbi olfactorii coupled with smaller tecta optica than their group-reared conspecifics, irrespectively of population origin. Evolutionary trade-offs between olfactory and visual centres of the primate brain have been shown at the interspecific level (Barton *et al.* 1995; Barton & Harvey 2000), but not in fish (Van Staaden *et al.* 1995; Huber *et al.* 1997). Our results support the existence of such a trade-off at the ontogenetic level: fish in a certain treatment not only enhanced the growth of the more-used structure, but also reduced the less-used one. These responses make sense considering the extremely high cost of developing and maintaining brain tissue (Aiello & Wheele 1995).

In summary, the results demonstrate that social environment—i.e. solitude versus membership of a group of conspecifics—has a marked effect on the development of the nine-spined stickleback brain. Individually reared pond fish developed relatively larger brains than their group-reared conspecifics from the same populations, while no such effect (or rather a tendency towards the opposite) was detected in marine fish. This pattern might arise from the higher costs of sociality in pond fish than in marine fish, originating in the lower benefits of grouping and higher drive for intraspecific competition in pond than in marine nine-spined sticklebacks (Herczeg *et al.* 2009*a*,*b*). Furthermore, we found that individually kept fish developed larger bulbi olfactorii but smaller tecta optica than fish kept in groups, irrespective of population origin. This finding supports the contention that the relative size of certain brain parts is related to their relative importance. Our study provides the first evidence for habitat-specific difference in brain-size plasticity, and emphasizes the importance of social environment in shaping brain architecture, with special emphasis on the main neural sensory centres.

The experiments were done under the licence of the Helsinki University Animal Experimentation Committee.

We thank Victor Berger, Göran Englund, Tuomas Leinonen, Daniel Lussetti and Pirkko Siikamäki for helping us in organizing and executing the field sampling, and John Loehr for correcting English. Special thanks to the Oulanka Research Station and White Sea Biological Station for their help and support. We are highly indebted to Hans Hofmann and Pertti Panula for helping with practical issues related to the preparation and measurement of brains. A.G. was supported by a fellowship from CIMO (www.cimo.fi). G.H. and J.M. were supported by the Academy of Finland.

REFERENCES

Adar, E., Lotem, A. & Barnea, A. 2008 The effect of social environment on singing behavior in the zebra finch (*Taeniopygia guttata*) and its implication for neuronal recruitment. *Behav. Brain Res.* **187**, 178–184. (doi:10.1016/j.bbr.2007.09.011)

Aiello, L. C. & Wheele, P. 1995 The expensive-tissue hypothesis: the brain and digestive system in human and primate evolution. *Curr. Anthropol.* **36**, 199–221. (doi:10.1086/204350)

Bănărescu, P. & Paepke, H. J. 2001. *The freshwater fishes of Europe. Vol. 5/III*. Wiebelsheim, Germany: AULA-Verlag.

Barton, R. A. & Harvey, P. H. 2000 Mosaic evolution of brain structure in mammals. *Nature* **405**, 1055–1058. (doi:10.1038/35016580)

Barton, R. A., Purvis, A. & Harvey, P. H. 1995 Evolutionary radiation of visual and olfactory brain systems in primates, bats and insectivores. *Phil. Trans. R. Soc. Lond. B* **348**, 381–392. (doi:10.1098/rstb.1995.0076)

Biro, P. A. & Dingemanse, N. J. 2009 Sampling bias resulting from animal personality. *Trends Ecol. Evol.* **24**, 66–67. (doi:10.1016/j.tree.2008.11.001)

Birse, S. C., Leonard, R. B. & Coggeshall, R. E. 1980 Neuronal increase in various areas of the nervous system of the guppy, *Lebistes*. *J. Comp. Neurol.* **194**, 291–301. (doi:10.1002/cne.901940202)

Brown, C. & Braithwaite, V. A. 2005 Effects of predation pressure on the cognitive ability of the poeciliid *Brachyraphis episcope*. *Behav. Ecol.* **16**, 482–487. (doi:10.1093/beheco/ari016)

Brown, C., Davidson, T. & Laland, K. 2003 Environmental enrichment and prior experience improve foraging behaviour in hatchery-reared Atlantic salmon. *J. Fish Biol.* **63**, 187–196. (doi:10.1111/j.1095-8649.2003.00208.x)

Brown, C., Western, J. & Braithwaite, V. A. 2007 The influence of early experience on, and inheritance of, cerebral lateralization. *Anim. Behav.* **74**, 231–238. (doi:10.1016/j.anbehav.2006.08.014)

Burns, J. G. & Rodd, H. 2008 Hastiness, brain size and predation regime affect the performance of wild guppies in a spatial memory task. *Anim. Behav.* **76**, 911–922. (doi:10.1016/j.anbehav.2008.02.017)

Clarke, B. C. 1975 The contribution of ecological genetics to evolutionary theory: detecting the direct effects of natural selection on particularly polymorphic loci. *Genetics* **79**, 101–113.

Diamond, M. C., Law, F., Rhodes, H., Lindner, B., Rosenzweig, M. R., Krech, D. & Bennett, E. L. 1966 Increases in cortical depth and glia numbers in rats subjected to enriched environment. *J. Comp. Neurol.* **128**, 117–126. (doi:10.1002/cne.901280110)

Draganski, B. & May, B. 2008 Training-induced structural changes in the adult human brain. *Behav. Brain Res.* **192**, 137–142. (doi:10.1016/j.bbr.2008.02.015)

Dunbar, R. I. M. & Shultz, S. 2007 Evolution in the social brain. *Science* **317**, 1344–1347. (doi:10.1126/science.1145463)

Endler, J. A. 1986 *Natural selection in the wild*. Princeton, NJ: Princeton University Press.

Font, E., Desfilis, E., Pérez-Cañellas, M. M. & Garcıa-Verdugo, J. M. 2001 Neurogenesis and neuronal regeneration in the adult reptilian brain. *Brain Behav. Evol.* **58**, 276–295. (doi:10.1159/000057570)

Foster, S. A. 1999 The geography of behaviour: an evolutionary perspective. *Trends Ecol. Evol.* **14**, 190–195. (doi:10.1016/S0169-5347(98)01577-8)

Fowler, D. C., Liu, Y., Ouimet, C. & Wang, Z. 2002 The effects of social environment on adult neurogenesis in the female prairie vole. *J. Neurobiol.* **51**, 115–128. (doi:10.1002/neu.10042)

Garamszegi, L. Z. & Eens, M. 2004 Brain space for a learned task: strong intraspecific evidence for neural correlates of singing behavior in songbirds. *Brain Res. Rev.* **44**, 187–193. (doi:10.1016/j.brainresrev.2003.12.001)

Girvan, J. R. & Braithwaite, V. A. 1998 Population differences in spatial learning in three-spine sticklebacks. *Proc. R. Soc. Lond. B* **265**, 913–918. (doi:10.1098/rspb.1998.0378)

Gonzalez-Voyer, A., Winberg, S. & Kolm, N. 2009 Social fishes and single mothers: brain evolution in African cichlids. *Proc. R. Soc. B* **276**, 161–167. (doi:10.1098/rspb.2008.0979)

Gould, E., Tanapat, P., Hastings, N. B. & Shors, T. J. 1999 Neurogenesis in adulthood: a possible role in learning. *Trends Cogn. Sci.* **3**, 186–192. (doi:10.1016/S1364-6613(99)01310-8)

Hastings, N. B., Tanapat, P. & Gould, E. 2000 Comparative views of adult neurogenesis. *Neuroscientist* **6**, 315–325.

Hastings, N. B., Tanapat, P. & Gould, E. 2001 Neurogenesis in the adult mammalian brain. *Clin. Neurosci. Res.* **1**, 175–182. (doi:10.1016/S1566-2772(01)00003-2)

Herczeg, G., Gonda, A. & Merilä, J. 2009*a* Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *J. Evol. Biol.* **22**, 544–552. (doi:10.1111/j.1420-9101.2008.01674.x)

Herczeg, G., Gonda, A. & Merilä, J. 2009*b* The social cost of shoaling covaries with predation risk in nine-spined stickleback (*Pungitius pungitius*) populations. *Anim. Behav.* **77**, 575–580. (doi:10.1016/j.anbehav.2008.10.023)

Huber, R., van Staaden, M., Kaufman, L. S. & Liem, K. F. 1997 Microhabitat use, trophic patterns and the evolution of brain structure in African cichlids. *Brain Behav. Evol.* **50**, 167–182. (doi:10.1159/000113330)

Kahilainen, K., Malinen, T., Tuomaala, A. & Lehtonen, H. 2004 Diel and seasonal habitat and food segregation of three sympatric *Coregonus lavaretus* forms in a subarctic lake. *J. Fish. Biol.* **64**, 418–434. (doi:10.1111/j.0022-1112.2004.00307.x)

Kempermann, G., Kuhn, H. G. & Gage, F. H. 1997 More hippocampal neurons in adult mice living in enriched environment. *Nature* **386**, 493–495. (doi:10.1038/386493a0)

Kihslinger, R. L. & Nevitt, G. A. 2006 Early rearing environment impacts cerebellar growth in juvenile salmon. *J. Exp. Biol.* **209**, 504–509. (doi:10.1242/jeb.02019)

Kihslinger, R. L., Lema, S. C. & Nevitt, G. A. 2006 Environmental rearing conditions produce forebrain differences in wild Chinook salmon *Oncorhynchus tshawytscha*. *Comp. Biochem. Physiol. A.* **145**, 145–151. (doi:10.1016/j.cbpa.2006.06.041)

Kotrschal, K., van Staaden, M. J. & Huber, R. 1998 Fish brains: evolution and environmental relationships. *Rev. Fish. Biol. Fish.* **8**, 373–408. (doi:10.1023/A:1008839605380)

Krause, J. & Ruxton, G. D. 2002 *Living in groups*. Oxford, UK: Oxford University Press.

Lefebvre, L., Whittle, P., Lascaris, E. & Finkelstein, A. 1997 Feeding innovation and forebrain size in birds. *Anim. Behav.* **53**, 549–560. (doi:10.1006/anbe.1996.0330)

Lema, S. C., Hodges, M. J., Marchetti, M. P. & Nevitt, G. A. 2005 Proliferation zones in the salmon telencephalon and evidence for environmental influence on proliferation rate. *Comp. Biochem. Physiol. A* **141**, 327–335. (doi:10.1016/j.cbpb.2005.06.003)

Lipkind, D., Nottebohm, R., Rado, R. & Barnea, A. 2002 Social change affects the survival of new neurons in the forebrain of adult songbirds. *Behav. Brain Res.* **133**, 31–43. (doi:10.1016/S0166-4328(01)00416-8)

Lisney, T. J., Bennett, M. B. & Collin, S. P. 2007 Volumetric analysis of brain areas indicates a shift in sensory orientation during development in the deep-sea grenadier *Coryphaenoides armatus*. *Raffl. Bull. Zool.* **14**, 7–15.

Mackney, P. A. & Hughes, R. N. 1995 Foraging behaviour and memory window in sticklebacks. *Behaviour* **132**, 1241–1253. (doi:10.1163/156853995X00559)

McGuigan, K., Chenoweth, S. F. & Blows, M. W. 2005 Phenotypic divergence along lines of genetic variance. *Am. Nat.* **165**, 32–43. (doi:10.1086/426600)

Mohammed, A. H., Zhu, S. W., Darmopil, S., Hjerling-Leffler, J., Ernfors, P., Winblad, B., Diamond, M. C., Eriksson, P. S. & Bogdanovic, N. 2002 Environmental enrichment and the brain. *Prog. Brain Res.* **138**, 109–133. (doi:10.1016/S0079-6123(02)38074-9)

Nelson, D. A., Marler, P. & Palleroni, A. 1995 A comparative approach to vocal learning: intraspecific variation in the learning process. *Anim. Behav.* **50**, 83–97. (doi:10.1006/anbe.1995.0223)

Nilsson, M., Perfilieva, E., Johansson, U., Orwar, O. & Eriksson, P. S. 1999 Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. *J. Neurobiol.* **39**, 569–578. (doi:10.1002/(SICI)1097-4695(19990615)39:4<569::AID-NEU10>3.0.CO;2-F)

Northcutt, R. G., Neary, T. J. & Senn, D. G. 1978 Observations on the brain of coelacanth *Latimeria chalumnae*: external anatomy and quantitative analysis. *J. Morph.* **155**, 181–192. (doi:10.1002/jmor.1051550205)

Perez-Barberia, F. J., Shultz, S. & Dunbar, R. I. M. 2007 Evidence for coevolution of sociality and relative brain size in three orders of mammals. *Evolution* **61**, 2811–2821. (doi:10.1111/j.1558-5646.2007.00229.x)

Pitcher, T. J. & Parrish, J. K. 1993 Functions of shoaling behaviour in teleosts. In *Behaviour of teleost fishes* (ed. T. J. Pitcher), pp. 363–439, 2nd edn. London, UK: Chapman & Hall.

Pollen, A. A., Dobberfuhl, A. P., Scace, J., Igulu, M. M., Renn, S. C. P., Shumway, C. A. & Hofmann, H. A. 2007 Environmental complexity and social organization sculpt the brain in Lake Tanganyikan cichlid fish. *Brain Behav. Evol.* **70**, 21–39. (doi:10.1159/000101067)

Pravosudov, V. V., Kitaysky, A. S. & Omanska, A. 2006 The relationship between migratory behaviour, memory and the hippocampus: an intraspecific comparison. *Proc. R. Soc. B* **273**, 2641–2649. (doi:10.1098/rspb.2006.3624)

Raymond, P. A. & Easter Jr, S. S. 1983 Postembryonic growth of the optic tectum in goldfish. I. Location of germinal cells and numbers of neurons produced. *J. Neurosci.* **3**, 1077–1091.

Reader, S. M. & Laland, K. M. 2002 Social intelligence, innovation, and enhanced brain size in primates. *Proc. Natl Acad. Sci. USA* **99**, 4436–4441. (doi:10.1073/pnas.062041299)

Reh, T. A. & Fischer, A. J. 2001 Stem cells in the vertebrate retina. *Brain Behav. Evol.* **58**, 296–305. (doi:10.1159/000057571)

Rohlf, F. J. 2002 tpsDig, digitize landmarks and outlines, version 1.37. Department of Ecology and Evolution, State University of New York at Stony Brook.

Rosenzweig, M. R. & Bennett, E. L. 1969 Effects of differential environments on brain weights and enzyme activities in gerbils, rats, and mice. *Dev. Psychobiol.* **2**, 87–95. (doi:10.1002/dev.420020208)

Schluter, D. & Nagel, L. M. 1995 Parallel speciation by natural selection. *Am. Nat.* **146**, 292–301. (doi:10.1086/285799)

Tramontin, A. D. & Brenowitz, E. A. 2000 Seasonal plasticity in adult brain. *Trends Neurosci.* **23**, 251–258. (doi:10.1016/S0166-2236(00)01558-7)

Van Praag, H., Kempermann, G. & Cage, F. H. 2000 Neural consequences of environmental enrichment. *Nat. Rev.* **1**, 191–198. (doi:10.1038/35044558)

Van Staaden, M., Huber, R., Kaufman, L. & Liem, K. 1995 Brain evolution in cichlids of the African Great Lakes: brain and body size, general patterns and evolutionary trends. *Zoology* **98**, 165–178.

Zupanc, G. K. H. 2001 Adult neurogenesis and neuronal regeneration in the central nervous system of teleost fish. *Brain Behav. Evol.* **58**, 250–275. (doi:10.1159/000057569)

Zupanc, G. K. H. 2006 Neurogenesis and neuronal regeneration in the adult fish brain. *J. Comp. Physiol. A* **192**, 649–670. (doi:10.1007/s00359-006-0104-y)

Zupanc, G. K. H. & Horschke, I. 1995 Proliferation zones in the brain of adult gymnotiform fish—a quantitative mapping study. *J. Comp. Neurol.* **353**, 213–233. (doi:10.1002/cne.903530205)

BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

Population variation in brain size of nine-spined sticklebacks (*Pungitius pungitius*) - local adaptation or environmentally induced variation?

Abigél Gonda*, Gábor Herczeg and Juha Merilä

Abstract

Background: Most evolutionary studies on the size of brains and different parts of the brain have relied on interspecific comparisons, and have uncovered correlations between brain architecture and various ecological, behavioural and life-history traits. Yet, similar intraspecific studies are rare, despite the fact that they could better determine how selection and phenotypic plasticity influence brain architecture. We investigated the variation in brain size and structure in wild-caught nine-spined sticklebacks (*Pungitius pungitius*) from eight populations, representing marine, lake, and pond habitats, and compared them to data from a previous common garden study from a smaller number of populations.

Results: Brain size scaled hypo-allometrically with body size, irrespective of population origin, with a common slope of 0.5. Both absolute and relative brain size, as well as relative telencephalon, optic tectum and cerebellum size, differed significantly among the populations. Further, absolute and relative brain sizes were larger in pond than in marine populations, while the telencephalon tended to be larger in marine than in pond populations. These findings are partly incongruent with previous common garden results. A direct comparison between wild and common garden fish from the same populations revealed a habitat-specific effect: pond fish had relatively smaller brains in a controlled environment than in the wild, while marine fish were similar. All brain parts were smaller in the laboratory than in the wild, irrespective of population origin.

Conclusion: Our results indicate that variation among populations is large, both in terms of brain size and in the size of separate brain parts in wild nine-spined sticklebacks. However, the incongruence between the wild and common garden patterns suggests that much of the population variation found in the wild may be attributable to environmentally induced phenotypic plasticity. Given that the brain is among the most plastic organs in general, the results emphasize the view that common garden data are required to draw firm evolutionary conclusions from patterns of brain size variability in the wild.

Background

During the past few decades, studies on diverse taxa have demonstrated that both absolute and relative brain size, as well as absolute and relative sizes of different brain parts, are highly variable and correlate with several environmental factors [mammals e.g. [1-3], birds e.g. [4,5] and fishes e.g. [6,7]]. Most of these studies, which form the basis of our current knowledge about brain size evolution, have used correlative approaches at the interspecific level. However, several recent studies have investigated differences in brain architecture among populations of the same species [8-17]. By using inter-population comparisons, microevolutionary processes can be investigated explicitly because most populations are likely to be found in the environment that actually shaped their brains. This is an unlikely situation in the case of comparisons based on species, which might have gone through adaptive divergence after splitting from common ancestor. An additional benefit from interpopulation comparisons as compared to interspecific comparisons is that the former allow adopting approaches to separate the relative roles of genetic drift and natural selection on observed differentiation [e.g. [18,19]]. Further, even fewer studies have compared brains of individuals from different populations reared under standardized settings to exclude the possible effects of phenotypic plasticity [but see: [10,12]]. This is surprising considering the fact that phenotypic plasticity in overall brain size, in addition to the size of different brain regions, has often been demonstrated [e.g. seasonality: [20,21]; spatial learning: [22,23], environmental heterogeneity: [24,25]]. Moreover, how this plasticity might influence population and species comparisons in terms of neural architecture has yet to be explored. Therefore, direct comparisons of patterns based on data collected from wild populations with those based on data from standardized common garden settings are needed to establish if any evolutionary inferences can be made from wild collected data in such a highly plastic organ as the brain.

The nine-spined stickleback (*Pungitius pungitius*) is an excellent model species for intraspecific comparative studies and exploring adaptive divergence. It occupies markedly different habitats, ranging from marine environments through large lakes to isolated ponds wherein they are often the only fish species present [e.g. [26]]. Hence, large differences can be found both in biotic (e.g. diversity of prey, competitors and predators) and abiotic (e.g. habitat structure) habitat components. These differences are expected to impose different selection pressures on complex behaviours and memory, and thus, also on the neural architecture. This is especially true in light of the high energetic costs of developing and maintaining large brains [27]. Our recent studies, utilizing common garden reared nine-spined sticklebacks, have demonstrated genetically-based and habitat-related divergence in (i) size of different brain parts [12] and (ii) brain plasticity in response to the social environment [13]. However, patterns found in the wild have not been reported, and the fit between patterns of variation in common garden and wild collected data has never been tested.

Brain size scales allometrically with body size, both on ontogenetic and on evolutionary scales [e.g. [28-32]]. The slope of the allometric relationship between brain size to body size (both variables plotted on a logarithmic scale) is higher in prenatal than adult stages in mammals [28]. Furthermore, the slope of this relationship tends to be steeper at higher taxonomic ranks [ca. 0.75 across mammalian orders; e.g. [29,30]] compared to closely related species, or in intraspecific comparisons (ca. 0.2-0.5; [31,32]). Although some intraspecific studies in brain-body size allometry exist [e.g. [33]], only very few investigations have been conducted within a single vertebrate species, perhaps due to a lack of sufficient within-species size variation among adults. Since nine-spined sticklebacks living in ponds have repeatedly evolved into giants [34,35], the species (representing tenfold body weight differences between adult individuals) also provides an excellent model to study intraspecific brain-body size allometry.

Our aim was to explore population divergence in brain size and in the size of different parts of the brain (*viz*. telencephalon, optic tectum, cerebellum, hypothalamus) in wild-caught nine-spined sticklebacks from different habitats, and to compare the observed patterns with previously reported common garden results [12]. We sampled fish from eight Fennoscandian populations (Figure 1) originating from three habitat types (*viz*. marine, lake and pond environments) to test (i) for differences in the size of the brain and different brain parts among wild populations, and (ii) whether observed differences were habitat specific. Furthermore, to establish whether data collected from the wild can be used for evolutionary inference, (iii) we tested whether data collected from the wild and common garden experiments for fish originating from the same populations are concurrent, and if not, (iv) whether observed differences are population- or habitat-specific. We expected that the higher biotic and abiotic variability of marine and lake environments as compared to pond environments have selected for relatively large brains. We also expected to find habitat-dependent differences in brain parts important in perception, learning and spatial memory, and that the stimulus-poor laboratory environment would reduce the brains of common garden fish compared to

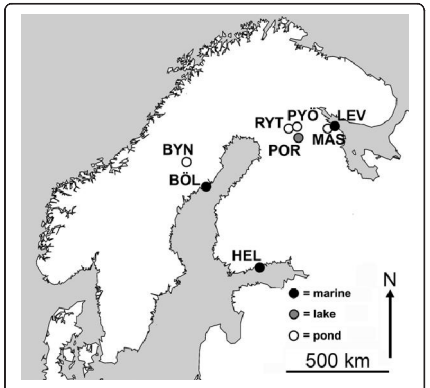


Figure 1 Map of the sampling localities. BÖL = Bölesviken, Baltic Sea, Sweden; HEL = Helsinki, Baltic Sea, Finland; LEV = Levin Navolok Bay, White Sea, Russia; POR = Porontima, Finland; BYN = Bynästjärnen, Sweden; PYÖ = Pyöreälampi, Finland; RYT = Rytilampi, Finland; MAS = Mashinnoje, Russia.

* Correspondence: abigel.gonda@helsinki.fi
Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, PO Box 65, FI-00014, Helsinki, Finland

their wild conspecifics. We also explored the brain-body size allometric relationship in fish from different populations, expecting hypoallometry with a relatively shallow slope (<0.5).

Results

Variation in absolute brain size

Dissected brains were fixed and photographed under standardized conditions. Absolute brain size was estimated from measurements taken from digital photographs (dorsal, lateral and ventral views) by using the ellipsoid model [12,36]. General Linear Model (GLM) results revealed a significant population effect in absolute brain size ($F_{7, 112} = 153.68$, $P < 0.001$). Average brain sizes of marine and lake fish were similar, but smaller than those of pond fish, the latter also being highly variable (Figure 2). General Linear Mixed Model (GLMM) analyses revealed a significant habitat ($F_{1, 5} = 11.84$, $P = 0.018$) and non-significant population within habitat effect ($Z = 1.55$, $P = 0.12$). Pond fish had brains almost twice as large as marine fish (Least Squares [LS] mean ± Standard Error [SE]: marine = 18.59 ± 3.43 mm^3; pond = 34.22 ± 2.97 mm^3).

Brain size co-varied with body weight, but independently of population origin and standard length (GLM; population: $F_{7, 96} = 1.50$, $P = 0.18$; log body weight: $F_{1, 96} = 7.63$, $P = 0.007$; log standard length: $F_{7, 96} = 0.52$, $P = 0.47$; population × log body weight: $F_{7, 96} = 0.79$, $P = 0.60$; population × log standard length: $F_{7, 96} = 1.48$, $P = 0.18$). The log brain size - log body weight regression revealed hypoallometry (i.e. both $\beta = 0$ and $\beta = 1$ were rejected: $R^2 = 0.88$, $\beta = 0.50$, SE[β] = 0.02, $P < 0.001$; Figure 3), indicating that brain size increased at half the rate of body size.

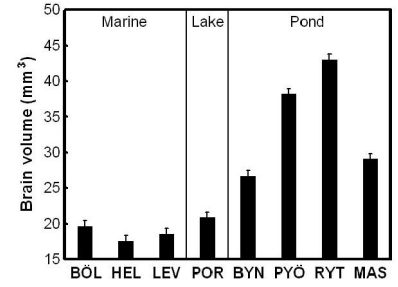


Figure 2 Population variation in absolute brain size in nine-spined sticklebacks. Means ± SE are shown. For population abbreviations, see Fig. 1.

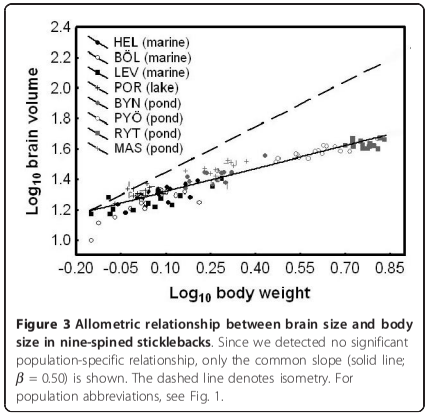


Figure 3 Allometric relationship between brain size and body size in nine-spined sticklebacks. Since we detected no significant population-specific relationship, only the common slope (solid line; $\beta = 0.50$) is shown. The dashed line denotes isometry. For population abbreviations, see Fig. 1.

Variation in relative brain size and brain part size

To study variation in relative brain size, we corrected the absolute brain size estimates to body length and body weight. We detected significant differences in relative brain size both at the habitat (GLMM; habitat: $F_{1, 6.89} = 10.38$, $P = 0.015$; log standard length: $F_{1, 57.02} = 0.27$, $P = 0.54$; log body weight: $F_{1, 93.48} = 28.82$, $P < 0.001$; population [habitat]: $Z = 0.95$, $P = 0.34$), and population level (GLM; population: $F_{7, 110} = 10.79$, $P < 0.001$; log standard length: $F_{1, 110} = 0.02$, $P = 0.89$; log body weight: $F_{1, 110} = 30.14$, $P < 0.001$). Pond (and the single lake) populations had larger relative brain sizes than marine populations (Figure 4a).

The sizes of different brain parts (telencephalon, optic tectum, cerebellum, hypothalamus) were also estimated from the digital photographs using the ellipsoid model [12,36]. We did not consider absolute size, but corrected our estimates with body length, body weight and absolute brain size. The multivariate GLM revealed a significant population effect on the relative sizes of different brain parts (population: Wilks' $\lambda_{28, 380} = 0.53$, $P < 0.001$; log body weight: Wilks' $\lambda_{4, 105} = 0.91$, $P = 0.043$; log standard length: Wilks' $\lambda_{4, 105} = 0.95$, $P = 0.024$; log brain volume: Wilks' $\lambda_{4, 105} = 0.15$, $P < 0.001$). Subsequent univariate tests indicated significant population differences in relative telencephalon ($F_{7, 108} = 3.53$, $P = 0.002$), optic tectum ($F_{7, 108} = 2.81$, $P = 0.010$), and cerebellum ($F_{7, 108} = 2.59$, $P = 0.016$) sizes, but not in hypothalamus ($F_{7, 108} = 1.47$, $P = 0.18$) size (Figure 4b-d). Our GLMMs revealed that neither optic tectum (habitat: $F_{1, 10.17} = 0.44$, $P = 0.52$; log standard length: $F_{1, 59.62} = 0.26$, $P = 0.61$; log body weight: $F_{1, 88.93} = 0.50$, $P = 0.48$; log brain volume: $F_{1, 99.99} = 262.72$, $P < 0.001$; population[habitat]: $Z = 1.01$, $P = 0.31$) nor cerebellum (habitat: $F_{1, 10.02} = 0.03$, $P = 0.87$; log standard length: $F_{1, 64.12} = 0.35$, $P = 0.056$; log body weight: $F_{1, 93.92} = 7.16$, $P = 0.009$; log brain volume: $F_{1, 99.91} = 32.39$, $P < 0.001$; population[habitat]: $Z = 1.12$, $P = 0.26$) showed significant habitat-dependency in their relative size. However, in the case of telencephalon, the habitat effect approached significance (habitat: $F_{1, 9.08} = 3.95$, $P = 0.078$; log standard length: $F_{1, 61.39} = 5.11$, $P = 0.027$; log body weight: $F_{1, 93.10} = 1.37$, $P = 0.24$; log brain volume: $F_{1, 99.91} = 122.53$, $P < 0.001$; population [habitat]: $Z = 1.05$, $P = 0.29$). Marine fish tended to have larger telencephala than pond fish, while no systematic trend could be observed in the other brain parts (Figure 4b-d).

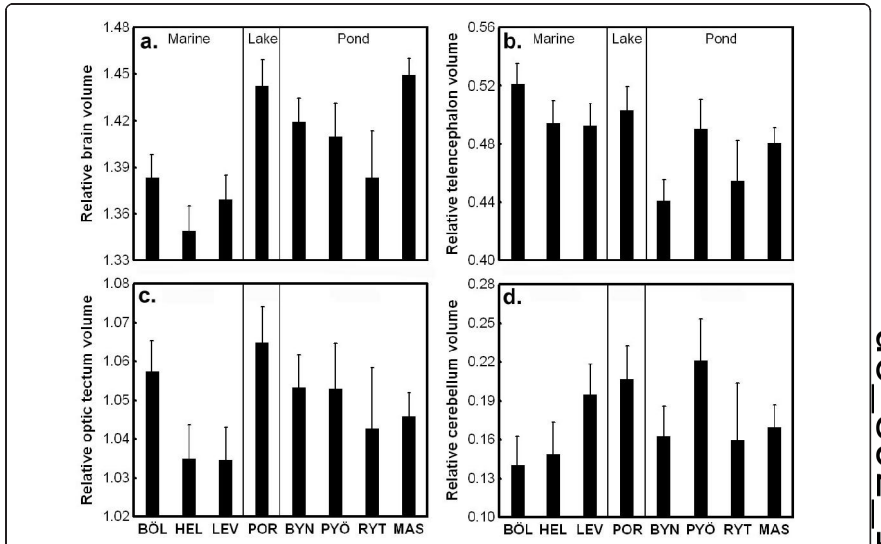


Figure 4 Population variation in relative brain size and relative size of different brain parts. Least Squares means ± SE are shown. Relative brain size is corrected for standard length and body weight while relative size of different brain parts are corrected for standard length, body weight and brain volume. For population abbreviations, see Fig.1.

Comparison of wild and common garden brains

We compared relative brain size and relative brain part size (see above) of fish from two marine (Helsinki, Baltic Sea and Levin Navolok Bay, White Sea; Figure 1) and two pond (Bynästjärnen, Sweden and Pyöreälampi, Finland; Figure 1) populations to data from the same populations reared in a common garden experiment [12]. Fish origin (i.e. wild-caught *vs*. common garden) had a habitat specific effect on relative brain size (GLMM; origin: $F_{1, 113.11} = 70.99$, $P < 0.001$; habitat: $F_{1, 2.49} = 0.65$, $P = 0.49$; origin × habitat: $F_{1, 113.83} = 38.36$, $P < 0.001$; log standard length: $F_{1, 105.99} = 5.75$, $P = 0.018$; log body weight: $F_{1, 113.95} = 42.12$, $P < 0.001$; population[habitat]: $Z = 0.84$, $P = 0.40$). The population-level GLM supported this result. It revealed a population-specific origin effect (origin: $F_{1, 110} = 70.41$, $P < 0.001$; population: $F_{3, 110} = 5.55$, $P = 0.001$; origin × population: $F_{3, 110} = 14.51$, $P < 0.001$; log standard length: $F_{1, 110} = 9.56$, $P = 0.003$; log body weight: $F_{1, 110} = 46.65$, $P < 0.001$). Relative brain size was similar for wild-caught and common garden marine fish, whereas pond fish had relatively larger brains in the wild than in the laboratory (Figure 5).

A multivariate GLM revealed significant, simple effects of population and origin on the relative size of brain parts, but no interaction between variables (origin: Wilks' $\lambda_{4, 106} = 0.74$, $P < 0.001$; population: Wilks' $\lambda_{12, 280.7} = 0.68$, $P < 0.001$; origin × population: Wilks' $\lambda_{12, 280.7} = 0.91$, $P = 0.57$; log standard length: Wilks' $\lambda_{4, 106} = 0.93$, $P = 0.12$; log body weight: Wilks' $\lambda_{4, 106} = 0.89$, $P = 0.012$; log brain volume: Wilks' $\lambda_{4, 106} = 0.107$, $P < 0.001$). All brain parts were affected by origin, as revealed by the subsequent univariate tests ($5.91 < F_{1, 109}$

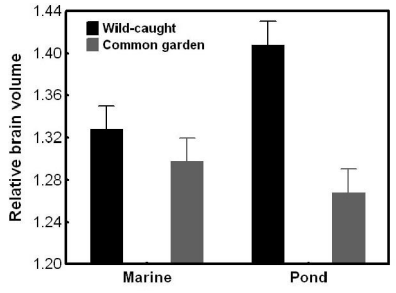


Figure 5 Habitat specific differences in relative brain size between wild-caught and common garden nine-spined sticklebacks. Least Squares means ± SE are shown. Relative brain size is corrected for standard length and body weight.

< 10.27, 0.002 <P < 0.017). Wild-caught fish had relatively larger telencephalon, optic tectum, cerebellum and hypothalamus volumes than their common garden reared conspecifics (Figure 6a-d).

Discussion

We showed that there is large variation in absolute brain volume, relative brain volume and relative volume of the telencephalon, optic tectum and cerebellum across wild nine-spined stickleback populations. Brain size patterns in the wild show habitat specificity both in absolute and relative scales: pond fish have larger brains than marine fish. Further, we found a marginally significant trend in the relative telencephalon size: marine fish tend to have larger telencephala than pond fish. The hypoallometric relationship between brain size and body size (slope = 0.5) is in accordance with a previous study on tropical fish [37]. We also found that wild-caught pond fish have larger brains than laboratory-reared pond fish, whereas no differences were observed between wild-caught and laboratory-reared marine conspecifics. The relative sizes of all brain parts were smaller in common garden than in the wild in all populations. These findings indicate that even though large brain size and brain part size variation exist in the wild, both in absolute and relative terms, patterns in nature may differ from those gathered in a standardized common garden and in some cases even in a habitat-dependent way. This strongly suggests that environmental effects on brain development can obscure and confound evolutionary inference based on purely phenotypic data collected from the wild. Hence, our results under-line the importance of not basing evolutionary inference on phenotypic patterns of brain size variation unless the environmental sources of variation have been controlled for - a point reinforced by other studies

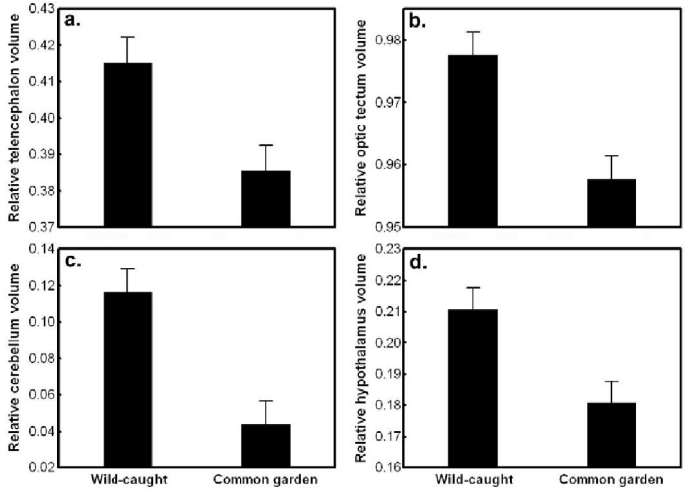


Figure 6 The relative size of different brain parts in wild-caught and common garden nine-spined sticklebacks. Least Squares means ± SE are shown. Relative size of different brain parts are corrected for standard length, body weight and brain volume.

focussing on differentiation in morphological and life history traits [38-40].

We found large habitat-specific population variation in absolute brain size: all marine populations (and a single lake population) had similarly sized brains that were nearly twofold smaller than those of pond fish. Within the pond habitat, there was large variation in average brain size. Although most studies have investigated only relative brain size variation (correcting brain size for variation in body size), absolute brain size can also account for differences in behaviour and/or habitat use. This is evident in comparisons of closely related species [see e.g. [32]], as the brains of these species tend to be more similar than those of distant taxa. Indeed, absolute brain size variation is routinely utilized in studies of primate and human evolution [e.g. [41]]. In general, increased brain size is attributable to an increase in neuron number, and not in neuron size [32]. Further, increases in absolute brain size result in decreased proportional connectivity [32]. Obviously, larger bodies need larger brains to be controlled at a similar level [32]. Hence, it is not surprising that pond populations that have evolved to giants [34,35] have also much larger brains than smaller sized marine or lake populations.

Previous findings have demonstrated a shallower hypoallometric slope at the intraspecific level and among closely related species than across broader taxonomic groups (mammals, intraspecific: 0.2-0.4, broad interspecific: 0.66, [28]; fishes, intraspecific: 0.44, intrafamiliar: 0.5, broad interspecific: 0.66, [37]). In accordance with these results we found a hypoallometric relationship between brain and body size with a slope = 0.5. In mammals, it has been demonstrated that on a broad evolutionary time scale, there has been greater net directional selection on brain size than on body size, while the short-term differentiation in brain *vs.* body size in closely related mammalian taxa has resulted from directional selection acting mostly on body size with changes in brain sizes being largely correlated responses [32]. Further, Gonzalez-Voyer et al. [42] demonstrated in Tanganyikan cichlids that even strongly correlated traits, such as brain and body size, can evolve independently from each other, and that body size may be under stronger selection than brain size during adaptive radiation. In the case of the nine-spined stickleback system, habitat-dependent body size diversification has been demonstrated [34,35], and body weight differences among these recently differentiated populations can be tenfold. Hence, it seems feasible to suggest that the observed brain size divergence might have been a correlated response to body-size divergence.

To assess body-size-independent brain size trends, we also investigated brain size differences relative to body size. Similarly to results for absolute brain size, and in contrast to our expectations, pond sticklebacks had relatively larger brains than marine sticklebacks. Intraspecific variation in relative brain size and brain architecture also appears to be strongly correlated with different ecological factors and/or life history traits. For example, environmental harshness has been shown to correlate positively with the size and neuron number of the brain region linked with memory storage (hippocampus) in the black-capped chickadee, *Poecile atricapillus*, a food caching species for which good memory can be essential for survival [14]. Garamszegi & Eens [8] found a positive correlation between song length and repertoire size and both relative and absolute volumes of different song nuclei. By comparing two subspecies of the white-crowned sparrow, the migratory *Zonotrichia leucophrys gambelii* and non-migratory *Z. l. nuttalli*, Pravosudov et al. [43] found that migratory subspecies had larger hippocampus and more hippocampal neurons. Habitat-independent, genetically based intraspecific variation in brain architecture has also been found both in wild guppy (*Poecilia reticulata*) populations reared in common environments [11], and in laboratory lines of the medaka (*Oryzias latipes;* [10]).

Marine nine-spined sticklebacks are members of a diverse fish fauna, and as such, are faced with numerous predators and interspecific competitors. In contrast, pond fish communities are much simpler. Structural heterogeneity in the pond environment is also much lower than that found in marine environments. These environmental factors are all known to be important in shaping brain evolution. For instance, predation pressure has been shown to affect brain size evolution in Mallorcan bovids [44], diet affected brain size evolution of bats [45], both environmental complexity and social features sculpt the brain architecture of cichlid fish [36], while living in large and socially complex groups is the most accepted hypothesis for the evolution of the extremely large brain of humans [46]. Hence, we expected selective pressures stemming from predation, interspecific competition, and habitat complexity to result in relatively larger brains in marine populations. Moreover, assuming that body size divergence (pond fish > marine/lake fish [34,35]) preceded correlated brain size divergence, we also expected pond fish to have similar or smaller brains, in relative terms, than marine or lake fish. Our previous common garden experiment based on a subset of the populations used here revealed no habitat-dependence in relative brain size [12]. Therefore, the pattern found in the current study (pond fish > marine fish) is highly unlikely to be a result of selection on brain size itself. Further, while we found no habitat-dependence in the common garden setting, strong population differentiation in relative brain size in a habitat-independent

way was detected (selective force unknown; [12]). Therefore, the plasticity resulting in the habitat-dependent wild *vs*. common garden difference cannot be habitat-specific itself. In a controlled laboratory experiment we found that group rearing had a negative effect on brain development in pond but not in marine fish [13]. Hence, the hypothesis that the aggressive, bold and anti-social pond fish [47,48] have larger relative brain sizes due to ontogenetic phenotypic plasticity as a response to fierce intraspecific competition must be rejected. Another possible explanation for larger relative brains in pond than in marine populations can be found from differences in ontogenetic allometry: pond fish living under negligible predation can become twice as old as marine fish [34], and an ontogenetic change in body *vs*. brain growth might explain this pattern. However, this issue requires further investigation.

Not only absolute and relative brain size, but also the relative size of different brain parts of nine-spined sticklebacks varied in the wild. Significant population differences were found in the relative sizes of the telencephalon, optic tectum and cerebellum. Further, we found marginally significant ($P < 0.08$) habitat-specificity in the relative size of the telencephalon, with marine fish tending to develop larger telencephala than pond fish. This is in accordance with results from our previous common garden study [12]. The telencephalon is larger in monogamous species, and shows a trend towards a positive correlation with rock size in the habitats in Tanganyikan lake cichlids [36], suggesting that both social and environmental heterogeneity may select for larger telencephalon. However, quite surprisingly, generalist limnetic populations of three-spined sticklebacks (*Gasterosteus aculeatus*) that use plankton as a main food source have larger telencephala than benthic-foraging populations of the same species as measured in samples from the wild [16]. The optic tectum is relatively larger in fish that prey on fish or other fast-moving prey, and clear water fishes develop larger optic tecta than species inhabiting turbid waters [6]. Cerebellum size correlates positively with the number of sympatric species in a fish community, and hypothalamus size is larger in monogamous than polygamous cichlids [36]. However, we did not find habitat specificity in the relative size of the optic tectum or the cerebellum, neither in the present, nor in the previous common garden study [12].

There are some incongruence between the present study and our previous work [12]. Here we found habitat-specific brain size divergence and population divergence in relative optic tectum size that was not seen in the common garden study. Only the habitat-dependence of relative telencephalon size found in the common garden study could be detected in the data from wild fish. A direct comparison between common garden and wild brains from the same populations revealed a habitat-dependent effect: pond (but not marine) fish had relatively larger brains in the wild than in the common garden. Further, the relative size of all brain parts was smaller in the laboratory than in the wild, perhaps due to a stimulus-poor environment during brain development. The most plausible explanation for the differences among common garden and wild data resides on phenotypic/ontogenetic plasticity in brain architecture. The potential for plastic responses to environmental heterogeneity is very high in fish [49-51]. Neurogenesis persists long into adulthood in fish [51-53] and contributes to lifelong growth of the brain. Hence, the fact that pond fish can live nearly twice as long as marine or lake fish may result in bias originating from plain ontogenetic plasticity or allometry. Furthermore, local random environmental variation may induce plasticity that could conceal genetic trends. Therefore, common garden studies seem to be of particular importance in studies of brain evolution. For instance, in this study system erroneous evolutionary conclusions could be drawn from the habitat-specificity (implying local adaptation) of relative brain size in the data from the wild given that observed differences cannot be reproduced under common garden conditions (showing that the differences are environmentally induced).

Finally, we showed that relative brain size and brain architecture are different between wild-caught and common garden sticklebacks from the same populations. The negative effect of domestication on brain size is well known both as a result of genetic adaptation and phenotypic plasticity [54,55]. In a recent paper, Burns et al. [56] demonstrated that laboratory rearing caused a significant decrease in the relative brain size of guppies (*P. reticulata*). Interestingly, we found that laboratory rearing had a negative effect on brain size in pond but not in marine nine-spined sticklebacks. The reason for this difference is unknown and warrants further investigations. We also found that all brain parts (corrected for both body and brain size) were smaller in common garden than in nature, a pattern congruent with general expectations. The reason for this can be a phenotypically plastic response to the comparatively stimulus poor laboratory environment.

Conclusion

In summary, we found large variation both in absolute and relative brain size, and brain architecture, among nine-spined sticklebacks in the wild. However, the patterns differed markedly from those found previously under standardized common garden settings, being most probably a result of environmental or age effects prevailing in the wild. Further, we found that the difference between wild or common garden samples can be habitat specific. Considering the extreme plasticity of the fish brain, drawing evolutionary inference from wild-collected material alone can be challenging, and easily misleading. To understand brain size/structure variation in the wild, more intraspecific, common garden based studies, especially those that attempt to separate genetic and environmental contributions to brain development are needed.

Methods

Sampling and husbandry

Adult fish were collected from eight populations during May and June of 2007. Three habitat types were covered: marine samples came from Helsinki (Baltic Sea, Finland), Bölesviken (Baltic Sea, Sweden) and Levin Navolok (White Sea, Russia); Rytilampi (Finland), Pyöreälampi (Finland) and Bynästjärnen (Sweden) are isolated ponds, and Iso-Porontima (Finland) is a lake (Figure 1). This region started to deglaciate around 8000 years ago [e.g. [57]], thus, the populations are younger than this. Fish were collected with seine nets and minnow traps. After collection, they were moved to the aquaculture facilities of the University of Helsinki. Prior to taking brain measurements, fish were kept in standardized environment for approximately three months: temperature (14°C) and photoperiod (12 h light, 12 h dark) were held constant, and feeding (*ad libitum*) with bloodworms (Chironomidae sp.) was similar for all population groups. We note that the ca. three months common garden keeping (to standardize body condition, which is highly variable during spring in nature) might have caused some plastic responses induced by the artificial environment. However, this effect is highly unlikely to be profound. The experiment was conducted under license of the Animal Experiment Board in Finland, reference number: STH379A.

Sampled habitat types differed markedly, both in terms of biotic and abiotic aspects. In marine and lake habitats, nine-spined sticklebacks belong to a diverse fish community consisting of a large number of potential fish predators and interspecific competitors. Conversely, ponds lack predatory fish, and interspecific competition is absent (Rytilampi and Bynästjärnen), or negligible (Pyöreälampi; where a few, small-sized whitefish [*Coregonus lavaretus*] were recently introduced). While predation by aquatic insects and cannibalism at very early stages might occur in both habitats, there are two facts indicating large differences in predation caused mortality at later-than-fry stages: pond fish (i) show marked reduction in their defensive body armour (pelvic apparatus; [58]) and (ii) have a much longer lifespan than marine fish (6-7 years *vs*. 3-4 years; [34]). The structural complexity of the marine and large lake environment exceeded that of the study ponds which exhibit very simple structure (*viz*. negligible vegetation, and only a few rocks or fallen logs at the bottom of the pond). Although we did not quantify the abundance of nine-spined sticklebacks, it was evident from catch numbers and relative effort that population densities in ponds exceed those in the marine environment.

Brain measurements

The entire procedure, from dissection through photography and measurement of whole brains and brain regions, followed exactly those outlined in Gonda et al. [12,13]. Fish (N = 15 per population) were euthanized with an overdose of MS 222 (tricaine methanesulfonate). Body weights were measured to the nearest 0.01 g with a digital balance and standard length (from the tip of the mouth to the end of the caudal peduncle) to the nearest 0.01 mm with digital callipers (for population variation in body weight and standard length see Additional file 1). Freshly dissected brains were fixed in 4% buffered formalin (0.1 M phosphate buffered saline) solution for 48 h. After fixation, digital photos were taken.

Width, height and length of the brain and four different parts of the brain - telencephalon, optic tectum, cerebellum and hypothalamus - were measured from the digital photographs using tpsDig 1.37 [59] software. They were defined as the greatest distance enclosed by the given structure. As the brains could not be cut off from the spinal cord at comparable positions in every individual, the end of the brain was defined as the perpendicular projection of the cerebellum on the medulla. We calculated the volume of the different brain parts according to the ellipsoid model [e.g. [60,36]]. Total brain volume was estimated with two different methods: first, with the equation of the ellipsoid model suggested by Pollen et al. [36]; second, we calculated brain volume by summing the volumes of the different parts. Both methods gave qualitatively similar results, thus, only the results from the ellipsoid model are reported. Repeatability (R) of the volume estimates was calculated from three repeated independent measurements of three independent photographs of a subsample of brains (N = 20). All volume variables were highly repeatable ($R > 0.86$, $P < 0.001$).

Analyses

Absolute brain size was compared among populations using a General Linear Model (GLM) with brain volume as dependent variable and population as fixed factor. To test the habitat effect directly, we also ran a General Linear Mixed Model (GLMM) with brain volume as a dependent variable, habitat type (marine vs. pond) as a fixed effect, and population, nested within habitat type,

as a random factor. Note that in this and the subsequent (see below) tests of habitat effects we excluded the single lake population and only compared three marine with four pond populations. To account for an allometric brain size - body size relationship, all metric variables were $\log_{10}$ (hereafter log) transformed. Because a GLM with log body weight as a dependent variable revealed population dependent patterns in the log standard length - log body weight relationship (population: $F_{7,\ 104} = 13.02$, $P < 0.001$; standard length: $F_{1,\ 104} = 152.73$, $P < 0.001$; population × standard length: $F_{7,\ 104} = 14.56$, $P < 0.001$), subsequent analyses of total brain size or brain part size included both log standard length and log body weight for size correction.

To study body - brain size allometry, we performed a GLM with log brain volume as the dependent variable, population as a fixed factor, and log standard length and log body weight as covariates, including factor × covariate interactions. We also ran a simplified GLM with only log body weight as a covariate, but the results remained qualitatively the same (data not shown). As only log body weight was significant in the model (see Results) the slope of the log brain size - log body weight correlation was determined by linear regression.

To assess relative brain size trends, we applied two approaches. First we ran a GLMM using log brain volume as the dependent variable, with habitat fixed and population nested within the habitat (random) factor, and log body weight and log standard length as covariates. Second, to compare populations directly, we ran a GLM with log brain volume as a dependent variable, population as a fixed factor and log body weight and log standard length as covariates. To compare the relative size of different brain parts, we ran a multivariate GLM with the brain parts as dependent variables, population as a fixed factor, and log body weight, log standard length and log brain volume as covariates. We note that random factors could not be properly computed in the multivariate context, and therefore, habitat effects could not be tested directly. In cases of a significant multivariate effect, related univariate tests were also performed. Upon significant univariate effect, we ran GLMMs testing for habitat effects with the given brain part as dependent variables, habitat as a fixed effect and population, nested within habitat, as a random factor, with log body weight, log standard length and log brain volume as covariates.

Finally, using data (N = 15 per population) from our previous common garden experiment [12] we compared a restricted set of populations for which we had data from both the wild and common garden. These populations were Helsinki (Baltic Sea, Finland), Levin Navolok Bay (White Sea, Russia), Bynästjärnen (pond, Sweden) and Pyöreälampi (pond, Finland). For these data we did not address absolute size. We compared relative brain volume by first running a GLMM with log brain volume as the dependent variable, habitat (marine *vs.* pond), origin (wild *vs.* common garden) and their interaction as fixed factors; population nested within habitat as a random factor, and log standard length and log body weight as covariates. Second, to compare populations, we ran a GLM with log brain volume as the dependent variable, population, origin and their interaction as fixed factors, and log standard length and log body weight as covariates. To compare the relative size of different brain parts, we ran a multivariate GLM with the log brain parts as dependent variables, population, origin and their interaction as fixed factors, and log standard length, log body weight and log brain volume as covariates. Upon significant multivariate effects, the related univariate tests were considered. Because we did not find significant population × origin interaction in the multivariate GLM (see Results), we did not address habitat × origin effects with GLMMs.

As in all GLMs and GLMMs testing for population or habitat divergence in relative brain volume or relative brain part volume the covariates were only used for correction, we did not include factor-covariate interactions. All statistical analyses were performed with the SPSS 18.0 for Windows package (SPSS Inc., Chicago, Illinois, USA).

Additional material

Additional file 1: Body size of the nine-spined sticklebacks (*Pungitius pungitius*) used in this study. Standard length and body weight of the nine-spined stickleback (*Pungitius pungitius*) individuals used in the present study in the different populations. Mean ± SD and the minimum - maximum range are presented.

Acknowledgements

We thank Victor Berger, Göran Englund, Tuomas Leinonen, Daniel Lussetti and Pirkko Siikamäki for helping us in organizing and executing the field sampling. We would also like to thank staff at the Oulanka Research Station and White Sea Biological Station. We are highly indebted to Hans Hofmann and Pertti Panula for helping with practical issues related to the preparation and measurement of brains. We thank Scott McCairns and Johan Kotze for their comments on the manuscript and for correcting the English. The study was financially supported by the Centre for International Mobility (AG) and the Academy of Finland (GH, JM).

Authors' contributions

AG collected data for the study, dissected the brains, took photos of them, measured the sizes of the brains and the sizes of the different brain parts and participated in the design of the study and writing of the manuscript. GH performed the statistical analyses, participated in the design of the study and helped in the writing of the manuscript. JM participated in the design and coordination of the study and helped in the writing of the manuscript. All authors read and approved the final manuscript.

Received: 29 November 2010 Accepted: 24 March 2011
Published: 24 March 2011

References

1. Harvey PH, Clutton-Brock TH, Mace GM: **Brain size and ecology in small mammals and primates.** *Proc Natl Acad Sci USA* 1980, **77**:4387-4389.
2. Clutton-Brock TH, Harvey PH: **Primates, brains and ecology.** *J Zool* 1980, **190**:309-323.
3. Marino L: **A comparison of encephalization between Odontocete Cetaceans and anthropoid primates.** *Brain Behav Evol* 1998, **51**:230-238.
4. Day LB, Westcott DA, Olster DH: **Evolution of bower complexity and cerebellum size in bowerbirds.** *Brain Behav Evol* 2005, **66(62)**:72.
5. Aviles JM, Garamszegi LZ: **Egg rejection and brain size among potential hosts of the common cuckoo.** *Ethology* 2007, **113**:562-572.
6. Kotrschal K, Van Staaden MJ, Huber R: **Fish brains: evolution and environmental relationships.** *Rev Fish Biol Fisheries* 1998, **8**:373-408.
7. Gonzalez-Voyer A, Winberg S, Kolm N: **Social fishes and single mothers: brain evolution in African cichlids.** *Proc R Soc B* 2009, **276**:161-167.
8. Garamszegi LZ, Eens M: **Brain space for a learned task: strong intraspecific evidence for neural correlates of singing behavior in songbirds.** *Brain Res Rev* 2004, **44**:187-193.
9. Karlen SJ, Krubitzer L: **Phenotypic diversity is the cornerstone of evolution: Variation in cortical field size within Short Tailed Opossums.** *J Comp Neur* 2006, **499**:990-999.
10. Ishikawa Y, Yoshimoto M, Yamamoto N, Ito H: **Different brain morphologies from different genotypes in a single teleost species, the medaka (*Oryzias latipes*).** *Brain Behav Evol* 1999, **53**:2-9.
11. Burns JG, Rodd H: **Hastiness, brain size and predation regime affect the performance of wild guppies in a spatial memory task.** *Anim Behav* 2008, **76**:911-922.
12. Gonda A, Herczeg G, Merilä J: **Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)?** *J Evol Biol* 2009, **22**:1721-1726.
13. Gonda A, Herczeg G, Merilä J: **Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain.** *Proc R Soc B* 2009, **276**:2085-2092.
14. Roth TC, Pravosudov VV: **Hippocampal volumes and neuron numbers increase along a gradient of environmental harshness: a large-scale comparison.** *Proc Roy Soc B* 2009, **276**:401-405.
15. Kolm N, Gonzalez-Voyer A, Brelin D, Winberg S: **Evidence for small scale variation in the vertebrate brain: mating strategy and sex affect brain size and structure in wild brown trout (*Salmo trutta*).** *J Evol Biol* 2009, **22**:2524-2531.
16. Park PJ, Bell MA: **Variation of telencephalon morphology of the threespine stickleback (*Gasterosteus aculeatus*) in relation to inferred ecology.** *J Evol Biol* 2010, **23**:1261-1277.
17. Chrispo E, Chapman LJ: **Geographic variation in phenotypic plasticity in response to dissolved oxygen in an African cichlid fish.** *J Evol Biol* 2010, **23**:2091-2103.
18. Merilä J, Crnokrak P: **Comparison of genetic differentiation at marker loci and quantitative traits.** *J Evol Biol* 2001, **14**:892-903.
19. Leinonen T, O'Hara R, Cano JM, Merilä J: **Comparative studies of quantitative trait and neutral marker divergence: a meta-analysis.** *J Evol Biol* 2008, **21**:1-17.
20. Nottebohm F: **A brain for all seasons: cyclical anatomical changes in song control nuclei of the canary brain.** *Science* 1981, **214**:1368-1370.
21. Tramontin AD, Brenowitz EA: **Seasonal plasticity in adult brain.** *Trends Neurosci* 2000, **23**:251-258.
22. Patel SN, Clayton NS, Krebs JR: **Spatial learning induces neurogenesis in the avian brain.** *Behav Brain Res* 1997, **89**:115-128.
23. Maguire EA, Gadian DG, Johnsrude IS, Good CD, Ashburner J, Frackowiak RSJ, Frith CD: **Navigation-related structural change in the hippocampi of taxi drivers.** *Proc Nat Acad Sci* 2000, **97**:4398-4403.
24. Marchetti MP, Nevitt GA: **Effects of hatchery rearing on brain structures of rainbow trout, *Oncorhynchus mykiss*.** *Env Biol Fish* 2003, **66**:9-14.
25. Kihslinger RL, Nevitt GA: **Early rearing environment impacts cerebellar growth in juvenile salmon.** *J Exp Biol* 2006, **209**:504-509.
26. Bănărescu PM, Paepke HJ: In *The Freshwater Fishes of Europe. Volume 5/III*. AULA-Verlag, Wiebelsheim; 2001.
27. Aiello LC, Wheele P: **The expensive tissue hypothesis - The brain and digestive system in human and primate evolution.** *Curr Anthrop* 1995, **36**:199-221.
28. Lande R: **Quantitative genetic analysis of multivariate evolution, applied to brain: body size allometry.** *Evolution* 1979, **33**:402-416.
29. Harvey PH, Bennett PM: **Brain size, energetics, ecology, and life history patterns.** *Nature* 1983, **306**:314-315.
30. Martin RD, Harvey PH: **Brain size allometry: ontogeny and phylogeny.** In *Size and scaling in primate biology*. Edited by: Jungers WL. New York; 1985:147-173.
31. Pagel MD, Harvey PH: **The taxon-level problem in the evolution of mammalian brain size: facts and artifacts.** *Am Nat* 1988, **132**:344-359.
32. Striedter GF: *Principles of Brain Evolution* Sunderland: Sinauer Associates; 2005.
33. Bauchot R, Platel R, Ridet JM: **Brain-body weight relationship in *Selachii*.** *Copeia* 1976, **2**:305-310.
34. Herczeg G, Gonda A, Merilä J: **Evolution of gigantism in nine-spined sticklebacks.** *Evolution* 2009, **63**:3190-3200.
35. Herczeg G, Gonda A, Merilä J: **Rensch's rule inverted - female-driven gigantism in nine-spined stickleback *Pungitius pungitius*.** *J Anim Ecol* 2010, **79**:581-588.
36. Pollen AA, Dobberfuhl AP, Scace J, Igulu MM, Renn SCP, Shumway CA, Hofmann HA: **Environmental complexity and social organization sculpt the brain in Lake Tanganyikan cichlid fish.** *Brain Behav Evol* 2007, **70**:21-39.
37. Bauchot R, Bauchot ML, Platel RRidet JM: **Brains of Hawaiian tropical fish; brain size and evolution.** *Copeia* 1977, **1/1977**:42-46.
38. Conover DO, Schultz ET: **Phenotypic similarity and the evolutionary significance of countergradient variation.** *Trends Ecol Evol* 1995, **10**:248-252.
39. Alho JS, Herczeg G, Söderman F, Laurila A, Jönsson KI, Merilä J: **Increasing melanism along a latitudinal gradient in a widespread amphibian: local adaptation, ontogenic or environmental plasticity?** *BMC Evol Biol* 2010, **10**:317.
40. Gienapp P, Teplitsky C, Alho JS, Mills JA, Merilä J: **Climate change and evolution: disentangling environmental and genetic responses.** *Mol Ecol* 2008, **17**:167-178.
41. Reader SM, Laland KM: **Social intelligence, innovation, and enhanced brain size in primates.** *Proc Natl Acad Sci USA* 2002, **99**:4436-4441.
42. Gonzalez-Voyer A, Winberg Kolm N: **Distinct evolutionary patterns of brain and body size during adaptive radiation.** *Evolution* 2009, **63**:2266-2274.
43. Pravosudov VV, Kitaysky AS, Omanska A: **The relationship between migratory behaviour, memory and the hippocampus: an intraspecific comparison.** *Proc Roy Soc B* 2006, **273**:2641-2649.
44. Köhler M, Moyà-Solà S: **Reduction of brain and sense organs in the fossil insular bovid *Myotragus*.** *Brain Behav Evol* 2004, **63**:125-140.
45. Jones KE, MacLarnon AM: **Affording larger brains: Testing hypotheses of mammalian brain evolution on bats.** *Am Nat* 2004, **164**:E20-E31.
46. Dunbar RIM, Shultz S: **Evolution in the social brain.** *Science* 2007, **317**:1344-1347.
47. Herczeg G, Gonda A, Merilä J: **Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*).** *J Evol Biol* 2009, **22**:544-552.
48. Herczeg G, Gonda A, Merilä J: **The social cost of shoaling covaries with predation risk in nine-spined stickleback (*Pungitius pungitius*) populations.** *Animal Behav* 2009, **77**:575-580.
49. Birse SC, Leonard RB, Coggeshall RE: **Neuronal increase in various areas of the nervous system of the guppy, *Lebistes*.** *J Comp Neurol* 1980, **194**:291-301.
50. Raymond PA, Easter SS Jr: **Postembryonic growth of the optic tectum in goldfish. I. Location of germinal cells and numbers of neurons produced.** *J Neurosci* 1983, **3**:1077-1091.
51. Zupanc GKH, Horschke I: **Proliferation zones in the brain of adult gymnotiform fish–a quantitative mapping study.** *J Comp Neurol* 1995, **353**:213-233.
52. Zupanc GKH: **Adult neurogenesis and neuronal regeneration in the central nervous system of teleost fish.** *Brain Behav Evol* 2001, **58**:250-275.
53. Zupanc GKH: **Neurogenesis and neuronal regeneration in the adult fish brain.** *J Comp Physiol A* 2006, **192**:649-670.
54. Kruska D: **Mammalian domestication and its effect on brain structure and behavior.** In *Intelligence and Evolutionary Biology*. Edited by: Jerison HJ, Jerison I. Springer-Verlag, Berlin; 1988:211-250.
55. Kihslinger RL, Lema SC, Nevitt GA: **Environmental rearing conditions produce forebrain differences in wild Chinook salmon *Oncorhynchus tshawytscha*.** *Comp Biochem Physiol A* 2006, **145**:145-151.

56. Bums JG, Saravanan A, Rodd FH: Rearing environment affects the brain size of guppies: lab-reared guppies have smaller brains than wild-caught guppes. *Ethology* 2009, 115:122-133.
57. Eronen M, Glückert G, Hatakka L, van der Plassche O, van der Plicht J, Rantala P: Rates of Holocene isostatic uplift and relative sea-level lowering of the Baltic in SW Finland based on studies of isolation contacts. *Boreas* 2001, 30:17-30.
58. Herczeg G, Tuttiainen M, Merilä J: Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. *Biol J Linn Soc* 2010, 101:413-416.
59. Rohlf FJ: 2002 tpsDig, digitize landmarks and outlines, version 1.37. Department of Ecology and Evolution, State University of New York at Stony Brook.
60. Huber R, van Staaden M, Kaufman LS, Liem KF: Microhabitat use, trophic patterns and the evolution of brain structure in African cichlids. *Brain Behav Evol* 1997, 50:167-182.

doi:10.1186/1471-2148-11-75
Cite this article as: Gonda *et al.*: Population variation in brain size of nine-spined sticklebacks (*Pungitius pungitius*) - local adaptation or environmentally induced variation? *BMC Evolutionary Biology* 2011 11:75.

biology letters
Evolutionary biology

Biol. Lett. (2012) **8**, 249–252
doi:10.1098/rsbl.2011.0837
Published online 28 September 2011

Brain development and predation: plastic responses depend on evolutionary history

Abigél Gonda*, Kaisa Välimäki, Gábor Herczeg and Juha Merilä

Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, PO Box 65, 00014, Finland
**Author for correspondence (abigel.gonda@helsinki.fi).*

Although the brain is known to be a very plastic organ, the effects of common ecological interactions like predation or competition on brain development have remained largely unexplored. We reared nine-spined sticklebacks (*Pungitius pungitius*) from two coastal marine (predation-adapted) and two isolated pond (competition-adapted) populations in a factorial experiment, manipulating perceived predatory risk and food supply to see (i) if the treatments affected brain development and (ii) if there was population differentiation in the response to treatments. We detected differences in plasticity of the bulbus olfactorius (chemosensory centre) between habitats: marine fish were not plastic, whereas pond fish had larger bulbi olfactorii in the presence of perceived predation. Marine fish had larger bulbus olfactorius overall. Irrespective of population origin, the hypothalamus was smaller in the presence of perceived predatory risk. Our results demonstrate that perceived predation risk can influence brain development, and that the effect of an environmental factor on brain development may depend on the evolutionary history of a given population in respect to this environmental factor.

Keywords: competition; predation; brain plasticity; brain size; *Pungitius*; stickleback

1. INTRODUCTION

The vertebrate brain is an organ with great capacity for plastic neuro-anatomical changes (e.g. [1–3]). Brain parts that are likely to be important in a particular context develop more than those of less importance [4]. Further, as the brain is the most expensive tissue to develop and maintain [5], energetic constraints should impose strong selection against non-adaptive modifications. Surprisingly, studies on the effect of biotic environment on brain development are scarce [6–8]. In particular, studies in which the effects of common ecological interactions have been tested—such as predation and competition—are notably absent from the literature [9,10].

The evolution of the brain has attracted more scientific interest than brain plasticity induced by ecologically relevant environmental factors. However, most studies of brain evolution have relied on interspecific comparisons, looking for correlations between brain architecture and fitness-related traits [11–13]. Studies of interpopulation differences in brain architecture from an evolutionary perspective have started to appear only recently [14–16]. Still, research integrating plasticity into the evolutionary perspective, by studying population variation in environmentally induced brain plasticity, is scarce [8,17].

In this study, we aimed to investigate the effects of two important ecological factors—predation pressure and food limitation—on the brain development of different nine-spined stickleback (*Pungitius pungitius*) populations. Nine-spine sticklebacks living in isolated ponds (zero piscine predation) are often more aggressive, bolder, long-living giants with reduced body armour and increased costs of group living when compared with marine sticklebacks facing high piscine predation [8,18–21]. These patterns suggest that marine nine-spined sticklebacks are mainly adapted to avoid predation, whereas pond fish are adapted to intraspecific competition. Here, we compared the brain development of sticklebacks from two pond and two coastal marine populations in the presence or absence of predator chemical cues, and subjected to two different levels of food supply.

2. MATERIAL AND METHODS

Adult nine-spined sticklebacks were collected in 2009 from two isolated ponds (Abbortjärn, Sweden, 64°29′ N, 19°26′ E; Pyöreälampi, Finland, 66°15′ N, 29°26′ E) and two low-salinity, coastal marine habitats from the Baltic Sea (Nyköping, Sweden, 58°39′ N; 17°06′ E; Helsinki, Finland, 60°13′ N, 25°11′ E). Pond habitats were small (less than 5 ha) and contained no sympatric predatory fish, whereas marine environments are characterized by more diverse ecological communities, replete with several piscine predators. Sample sites were separated by more than 500 km. Crosses (six to eight per population) were done *in vitro* during July and August. Fish were reared individually in 1.4 l plastic tanks within four Allentown Zebrafish Rack Systems (Aquaneering Inc., San Diego, CA, USA, hereafter 'rack'), each equipped with physical, chemical, biological and UV filters and a closed water circulation system. All rearing took place in freshwater. The photoperiod was set to 14 L : 10 D cycle (light : dark), and the water temperature was held constant at 12°C.

Fish were distributed into four treatment combinations of two perceived predation risk and two food levels in a full-factorial, randomized block design. The water reservoir of each rack was connected to a separate 150 l tank. In the predation treatment, two 10–15 cm long perch (*Perca fluviatilis*) were placed in two randomly chosen tanks while the other two tanks contained only water. Hence, in the predation treatment olfactory cues from a fish predator abundant in the Baltic Sea and Fennoscandian freshwater habitats were either present or absent. In the food treatment, predation treatments were randomly divided into high (two ad libitum feedings per day) and low (one ad libitum feeding per two days) food groups. Feeding was started with live brine shrimp nauplii (*Artemia* sp.), and was gradually changed to frozen bloodworms after 80 days. Each full-sib family was represented in each treatment combination.

At the age of 34 weeks—when fish approached adult size—individuals were euthanized by MS-222, photographed under standardized conditions, and weighed to the nearest 0.01 g. Standard length was measured from pictures using tpsDig v. 2.15 [22]. Brains of the fish were dissected, fixed in 4 per cent formalin—0.1 M phosphate-buffered saline solution, and photographed with a digital camera connected to a dissecting microscope from dorsal, lateral and ventral viewpoints. Size of the brain and five brain parts (*viz.* bulbus olfactorius, telencephalon, tectum opticum, cerebellum and hypothalamus) were measured from the digital photographs with tpsDig v. 2.15 [22] (see also the electronic supplementary material). The volume of the total brain and the different brain parts was estimated according to the ellipsoid model, which estimates volume based on the length, width and height of the object using correction factors [8,14,15].

In total, 187 brains were analysed. Some individuals could not be included owing to random mortality (mainly at early life stages), fish escaping the system and problematic dissections. Hence, family effects were not analysed, but rather it was assumed that sampled fish represented an unbiased sample of each source population's genetic pool. All metric variables were $\log_{10}$ transformed. We used general linear mixed models (GLMMs) to analyse variation in brain size and the size of different brain parts. The models were built with habitat (marine versus pond), sex, predation (presence versus absence of perceived predation risk) and food treatments (high versus low) as fixed effects, with population nested in habitat as random factor. Standard length and body weight were both added as covariates because the study populations differ in relative weight [8,14,15]. In the analyses of brain parts, we also added total brain size as a covariate. In all models, we included simple effects, and all two-way interactions between fixed factors. We note that sex was included in the models only to control for sex-related variation. As we focused on the habitat and treatment effects, sex effects will be reported, but not discussed here.

Electronic supplementary material is available at http://dx.doi.org/10.1098/rsbl.2011.0837 or via http://rsbl.royalsocietypublishing.org.

Received 20 August 2011
Accepted 9 September 2011

Table 1. Results of the GLMMs. *F*-statistics and degrees of freedom are shown. Note that sex and its interactions were only included in the model to control for sex-related variation, and they are not discussed further.

effect	total brain	bulbus olfactorius	telencephalon	tectum opticum	cerebellum	hypothalamus
habitat (H)	3.89 (1,2.23)	29.24* (1,3.15)	0.50 (1,2.37)	<0.01 (1,2.39)	2.64 (1,2.68)	0.96 (1,3.08)
predation (P)	<0.01 (1,170)	1.97 (1,170)	1.41 (1,169)	0.02 (1,169)	0.06 (1,170)	3.89[a] (1,171)
food (F)	0.34 (1,170)	0.65 (1,170)	0.79 (1,169)	0.59 (1,169)	0.38 (1,170)	0.01 (1,170)
sex (S)	124.54*** (1,171)	6.09* (1,171)	25.96*** (1,170)	3.27 (1,170)	2.44 (1,170)	8.56** (1,171)
H × P	0.02 (1,170)	4.14* (1,171)	0.11 (1,170)	0.10 (1,170)	0.03 (1,170)	0.40 (1,171)
H × F	2.62 (1,170)	0.05 (1,169)	0.02 (1,169)	0.27 (1,169)	0.02 (1,169)	0.04 (1,169)
P × F	2.09 (1,170)	0.28 (1,171)	0.23 (1,170)	1.86 (1,170)	0.32 (1,170)	0.10 (1,171)
S × H	0.54 (1,170)	0.30 (1,170)	0.14 (1,169)	0.26 (1,169)	0.49 (1,169)	0.32 (1,170)
S × P	0.03 (1,170)	0.56 (1,170)	1.02 (1,169)	0.13 (1,169)	0.07 (1,170)	0.01 (1,170)
S × F	0.23 (1,170)	7.52** (1,169)	0.02 (1,169)	0.11 (1,169)	0.07 (1,169)	0.15 (1,169)
length	0.97 (1,172)	3.87 (1,155)	0.29 (1,170)	1.76 (1,171)	0.60 (1,170)	1.15 (1,150)
weight	86.74*** (1,169)	1.91 (1,65)	0.44 (1,155)	1.29 (1,158)	5.24* (1,144)	1.32 (1,54.4)
total brain	—	45.80*** (1,158)	151.07*** (1,171)	303.12*** (1,171)	208.45*** (1,170)	74.75*** (1,153)

*$p > 0.05$; **$p > 0.01$; ***$p > 0.001$; [a]$p = 0.0502$.

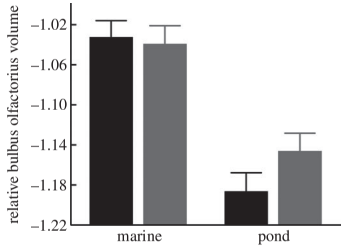


Figure 1. Effects of habitat of origin and perceived predation risk on bulbus olfactorius development. Least-squares means (from the GLMM) ± s.e. are shown. Black bars denote predator absent and grey bars denote predator present.

3. RESULTS

Brain plasticity was habitat-dependent (table 1): predation risk induced development of larger bulbi olfactorii in pond fish, but not in marine fish (figure 1). In general, marine fish had relatively larger bulbi olfactorii than pond fish (figure 1). Predation also had an effect on the development of the hypothalamus (table 1): independent of population, habitat or sex, fish developed smaller hypothalami in the presence of predator (figure 2). The food treatment did not affect brain development. The population effect was always non-significant ($p > 0.18$).

4. DISCUSSION

To date, the effect of predation on brain development has only been assessed in a single population of anurans [9,10]. Here, we found that perceived predation risk in the absence of actual contact with the predator has a significant—and sometimes habitat-dependent—effect on brain development in nine-spined sticklebacks. Surprisingly, while marine sticklebacks had relatively larger bulbi olfactorii than pond fish, perceived predation risk induced plastic modification in the bulbus olfactorius only in the latter. Our results suggest that in marine environments under constant predation risk, a large relative size of bulbus olfactorius might have become fixed, while in ponds, plasticity of the relatively small bulbi olfactorii occurred. Taken together, predation induced bulbus olfactorius enlargement both on the evolutionary and ontogenetic scales. As the brain is an extremely expensive tissue both to develop and maintain [5], the observed patterns support the conjecture that the bulbus olfactorius is an important sensory centre in predation avoidance. Given that predation-adapted sticklebacks are likely to represent the ancestral form, the fact that the plastic response appeared parallel to a decrease in bulbus olfactorius size in the piscine-predator free ponds warrants further investigations. We note that the predation treatment also reduced the aggression and risk-taking of our fish, demonstrating that sticklebacks identified olfactory cues from perch as predation risk [23].

Independent of population, habitat and sex, fish developed smaller hypothalami in the presence of predatory cues than in their absence. The hypothalamus has a wide range of functions [24]. For instance, it regulates reproductive behaviour [25], and it is also the centre of regulating feeding behaviour in fish [26]. Hence, based on our data, it is impossible to determine why perceived predation risk resulted in decreased hypothalamus size. However, considering

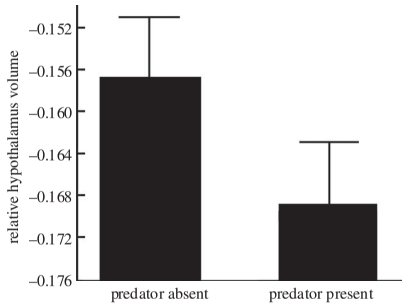


Figure 2. The effect of perceived predation risk on hypothalamus development. Least-squares means (from the GLMM) ± s.e. are shown.

that feeding behaviour can be regulated by different stress factors such as predation [26], and that nine-spined sticklebacks from the present experiment decreased their aggression and risk-taking levels in the presence of perceived predation risk [23], it seems possible that decreased hypothalamus size is somehow linked to the altered behavioural activity.

In summary, we found that perceived predation risk affected brain development, and that the effect can depend on a population's evolutionary history with predation. Available energy did not affect brain development. Interestingly, stickleback populations evolving under negligible predation had the ability to react to chemical predatory cues, while predation-adapted populations have evolved towards fixation of larger sized neural chemosensory centre (bulbus olfactorius). In the case of the hypothalamus, the negative effect of perceived predation risk affected all fish similarly, suggesting that the predation-induced reduction of functions regulated by the hypothalamus was general.

Our work was supported by the Academy of Finland (G.H. and J.M.), Finnish School in Wildlife Biology, Conservation and Management (A.G.) and Finnish Graduate School in Population Genetics (K.V.). The experiment was conducted under the license of the Finnish National Animal Experiment Board (Licence no. STH223A).

1 Kempermann, G., Kuhn, H. G. & Gage, F. H. 1997 More hippocampal neurons in adult mice living in enriched environment. *Nature* **386**, 493–495. (doi:10.1038/386493a0)
2 Tramontin, A. D. & Brenowitz, E. A. 2000 Seasonal plasticity in adult brain. *Trends Neurosci.* **23**, 251–258. (doi:10.1016/S0166-2236(00)01558-7)
3 Zupanc, G. K. H. 2001 Adult neurogenesis and neuronal regeneration in the central nervous system of teleost fish. *Brain Behav. Evol.* **58**, 250–275. (doi:10.1159/000057569)
4 Kihslinger, R. L. & Nevitt, G. A. 2006 Early rearing environment impacts cerebellar growth in juvenile salmon. *J. Exp. Biol.* **209**, 504–509. (doi:10.1242/jeb.02019)
5 Aiello, L. C. & Wheele, P. 1995 The expensive tissue hypothesis—the brain and digestive system in human and primate evolution. *Curr. Anthropol.* **36**, 199–221. (doi:10.1086/204350)
6 Fowler, D. C., Liu, Y., Ouimet, C. & Wang, Z. 2002 The effects of social environment on adult neurogenesis in the female prairie vole. *J. Neurobiol.* **51**, 115–128. (doi:10.1002/neu.10042)
7 Sørensen, C., Øverli, Ø., Summers, C. H. & Nilsson, G. E. 2007 Social regulation of neurogenesis in teleosts. *Brain Behav. Evol.* **70**, 239–246. (doi:10.1159/000105487)
8 Gonda, A., Herczeg, G. & Merilä, J. 2009 Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain. *Proc. R. Soc. B* **276**, 2085–2092. (doi:10.1098/rspb.2009.0026)
9 Gonda, A., Trokovic, N., Herczeg, G., Laurila, A. & Merilä, J. 2010 Predation- and competition-mediated brain plasticity in *Rana temporaria* tadpoles. *J. Evol. Biol.* **23**, 2300–2308. (doi:10.1111/j.1420-9101.2010.02066.x)
10 Trokovic, N., Gonda, A., Herczeg, G., Laurila, A. & Merilä, J. 2011 Brain plasticity over the metamorphic boundary: carry-over effect of larval environment on froglet brain development. *J. Evol. Biol.* **24**, 1380–1385. (doi:10.1111/j.1420-9101.2011.02275.x)
11 Garamszegi, L. Z. & Eens, M. 2004 The evolution of hippocampus volume and brain size in relation to food hoarding in birds. *Ecol. Lett.* **7**, 1216–1224. (doi:10.1111/j.1461-0248.2004.00685.x)
12 Sol, D., Bacher, S., Reader, S. M. & Lefebvre, L. 2008 Brain size predicts the success of mammal species introduced into novel environments. *Am. Nat.* **172**, S63–S71. (doi:10.1086/588304)
13 Gonzalez-Voyer, A., Winberg, S. & Kolm, N. 2009 Social fishes and single mothers: brain evolution in African cichlids. *Proc. R. Soc. B* **276**, 161–167. (doi:10.1098/rspb.2008.0979)
14 Gonda, A., Herczeg, G. & Merilä, J. 2009 Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? *J. Evol. Biol.* **22**, 1721–1726. (doi:10.1111/j.1420-9101.2009.01782.x)
15 Gonda, A., Herczeg, G. & Merilä, J. 2011 Population variation in brain size of nine-spined sticklebacks (*Pungitius pungitius*)—local adaptation or environmentally induced variation? *BMC Evol. Biol.* **11**, 75. (doi:10.1186/1471-2148-11-75)
16 Park, P. J. & Bell, M. A. 2010 Variation of telencephalon morphology of the threespine stickleback (*Gasterosteus aculeatus*) in relation to inferred ecology. *J. Evol. Biol.* **23**, 1261–1277. (doi:10.1111/j.1420-9101.2010.01987.x)
17 Crispo, E. & Chapman, L. J. 2010 Geographic variation in phenotypic plasticity in response to dissolved oxygen in an African cichlid fish. *J. Evol. Biol.* **23**, 2091–2103. (doi:10.1111/j.1420-9101.2010.02069.x)
18 Herczeg, G., Gonda, A. & Merilä, J. 2009 The social cost of shoaling covaries with predation risk in nine-spined stickleback (*Pungitius pungitius*) populations. *Anim. Behav.* **77**, 575–580. (doi:10.1016/j.anbehav.2008.10.023)
19 Herczeg, G., Gonda, A. & Merilä, J. 2009 Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *J. Evol. Biol.* **22**, 544–552. (doi:10.1111/j.1420-9101.2008.01674.x)
20 Herczeg, G., Gonda, A. & Merilä, J. 2009 Evolution of gigantism in nine-spined sticklebacks. *Evolution* **63**, 3190–3200. (doi:10.1111/j.1558-5646.2009.00781.x)
21 Herczeg, G., Turtiainen, M. & Merilä, J. 2010 Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. *Biol. J. Linn. Soc.* **101**, 403–416. (doi:10.1111/j.1095-8312.2010.01518.x)
22 Rohlf, F. J. 2006 *TPS software series* (distributed by the author). Stony Brook, NY: Department of Ecology and Evolution, State University of New York.
23 Herczeg, G. & Välimäki, K. In press. Intraspecific variation in behaviour: effects of evolutionary history, ontogenetic experience and sex. *J. Evol. Biol.* (doi:10.1111/j.1420-9101.2011.02371.x)
24 Kotrschal, K., Van Staaden, M. J. & Huber, R. 1998 Fish brains: evolution and environmental relationships. *Rev. Fish. Biol. Fish.* **8**, 373–408. (doi:10.1023/A:1008839605380)
25 White, S. A. & Fernald, R. D. 1993 Gonadotropin-releasing hormone-containing neurons change size with reproductive state in female *Haplochromis burtoni*. *J. Neurosci.* **13**, 434–441.
26 Kulczykowska, E. & Sánchez Vázquez, F. J. 2010 Neurohormonal regulation of feed intake and response to nutrients in fish: aspects of feeding rhythm and stress. *Aquacultural Res.* **41**, 654–667. (doi:10.1111/j.1365-2109.2009.02350.x)

Downloaded from rsbl.royalsocietypublishing.org on April 5, 2012

Biol. Lett. (2012)

JOURNAL OF Evolutionary Biology

doi: 10.1111/j.1420-9101.2011.02371.x

Intraspecific variation in behaviour: effects of evolutionary history, ontogenetic experience and sex

G. HERCZEG & K. VÄLIMÄKI

Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, Helsinki, Finland

Keywords:

behavioural evolution;
behavioural plasticity;
competition;
local adaptation;
phenotypic plasticity;
predation;
Pungitius;
stickleback.

Abstract

Geographical variation in behaviour within species is common. However, how behavioural plasticity varies between and within locally adapted populations is less studied. Here, we studied behavioural plasticity induced by perceived predation risk and food availability in pond (low predation – high competition) *vs.* coastal marine (high predation – low competition) nine-spined sticklebacks (*Pungitius pungitius*) reared in a common garden experiment. Pond sticklebacks were more active feeders, more risk-taking, aggressive and explorative than marine sticklebacks. Perceived predation risk decreased aggression and risk-taking of all fish. Food restriction increased feeding activity and risk-taking. Pond sticklebacks became more risk-taking than marine sticklebacks under food shortage, whereas well-fed fish behaved similarly. Among poorly fed fish, males showed higher drive to feed, whereas among well-fed fish, females did. Apart from showing how evolutionary history, ontogenetic experience and sex influence behaviour, the results provide evidence for habitat-dependent expression of adaptive phenotypic plasticity.

Introduction

Spatial environmental variation can cause corresponding spatial variation in the optimal phenotype, and as a consequence, environmental variation can drive phenotypic divergence between populations of the same species (Mayr, 1963; Endler, 1977). Such phenotypic divergence can emerge in at least two distinct ways: new superior phenotypes can emerge from a single genotype (phenotypic plasticity; e.g. West-Eberhard, 2003) or the allele frequencies can shift at the population level resulting in increased fitness (local adaptation; e.g. Kawecki & Ebert, 2004). Whereas the repeated occurrence of the same phenotypes in similar habitats can be interpreted to imply that the pattern is driven by natural selection (e.g. Clarke, 1975; Endler, 1986; McGuigan *et al.*, 2005), phenotypic plasticity alone can result in similar patterns. The role of phenotypic plasticity in local adaptation is widely discussed (Pigliucci *et al.*, 2006; Crispo, 2007, 2008; Fusco & Minelli, 2010; Pfennig *et al.*, 2010); however, our understanding as to how local adaptations affect the expression of phenotypic plasticity is still limited.

The presence of population-level variation in behaviour is well known (e.g. Foster, 1999; Foster & Endler, 1999; Gabor & Ryan, 2001). Predation commonly shapes behaviour (e.g. Giles & Huntingford, 1984; Riechert & Hedrick, 1990; Mathis *et al.*, 1993; Huntingford *et al.*, 1994; Storfer & Sih, 1998; Åbjörnsson *et al.*, 2004) and often influences many different behaviours at the same time. For instance, Magurran & Seghers (1991, 1994) reported that predator inspection, shoaling and aggression all covary with predation pressure in wild guppies (*Poecilia reticulata*). Habitat-dependent population divergence in behaviour has often been found to have a genetic basis (e.g. Breden *et al.*, 1987; Magurran, 1990; Brown *et al.*, 2007; Shaw *et al.*, 2007). Interestingly, ontogenetic experience can affect the expression of heritable variation in behaviour in a population-specific way (Dingemanse *et al.*, 2009). Behaviour is considered to be one of the most plastic phenotypic traits (e.g. West-Eberhard, 1989), and ontogenetic exposure to predation or food stress is a common factor resulting in plastic behavioural shifts (e.g. Tollerian & Harvell, 1999; Relyea, 2001; Biro *et al.*, 2005; Steiner, 2007). However, how behavioural plasticity induced by variation in certain environmental factors varies between populations adapted to differences in those environmental factors has only been addressed in a few systems, and the results are mixed (e.g. de Meester, 1993; Van Buskirk & Arioli, 2005; Salonen & Peuhkuri, 2007). Studies where more than one environmental factor and/or a number of different behaviours are assessed in parallel are especially lacking. This is surprising, given that one environmental factor might affect several behaviours and a certain behaviour might be affected by more than one environmental factor on both the evolutionary and ontogenetic scales.

The nine-spined stickleback (*Pungitius pungitius* Linnaeus) is a small teleost fish that is widely distributed in the Northern Hemisphere and can be found in a wide array of habitat types such as coastal marine environments, large lake or river systems, small creeks and ditches, and isolated ponds where they are often the only fish species (e.g. Bănărescu & Paepke, 2001; Östlund-Nilsson *et al.*, 2007). This habitat diversity provides an opportunity to study adaptive divergence in response to the biotic environment by comparing coastal marine or lake populations (high piscine predation) to pond populations (no piscine predation). Our previous work has revealed substantial habitat-dependent population divergence in morphological, life history and behavioural traits between coastal marine and pond populations (Gonda *et al.*, 2009a,b; Herczeg *et al.*, 2009a,b,c, 2010a,b, 2011); all patterns suggesting that coastal marine populations are adapted to high predation (e.g. small size, quick growth, low reproductive output and relatively strong body armour), whereas isolated pond populations are adapted to high intraspecific competition (e.g. giant size, slow growth, high reproductive output and reduced or absent body armour). With respect to behaviour, pond sticklebacks were found to be more aggressive, to be bolder and to have higher feeding activity than marine sticklebacks (Herczeg *et al.*, 2009a). These behavioural differences have profound effects: group living entails high developmental costs for pond fish, even in the absence of ecological or reproductive constraints, whereas no such cost was detected in marine fish (Gonda *et al.*, 2009b; Herczeg *et al.*, 2009c). However, nothing is known about how these different populations express behavioural plasticity towards variation in perceived predation risk or competition.

The goal of the present study was to investigate the behavioural plasticity of coastal marine and pond nine-spined stickleback populations reared under different levels of perceived predation risk and food supply. We aimed to disentangle the effects of population history, rearing environment and sex on a set of relevant behaviours. We hypothesized that coastal marine populations (high predation) will show a stronger response to the manipulation of perceived predation risk, whereas the pond populations (high competition) will show a stronger response towards food manipulation. We were also interested to see whether the patterns were different between the sexes, as we found sexual dimorphism in size and growth between habitats (females > males; Herczeg *et al.*, 2010b, 2011), suggesting different energy needs for the sexes. To this end, we studied feeding activity, risk-taking, aggression and exploration of fish from four marine and two pond populations reared in a common garden setting from hatching to adulthood in a factorial experiment with two predation (presence/absence) and two food (high/low) treatments.

Materials and methods

The common garden experiment

Adult nine-spined sticklebacks were collected from four coastal marine and two pond (surface area < 5 ha) populations (Fig. 1) before the onset of the reproductive season in 2009 with the aid of minnow traps and seine nets. Sticklebacks were transferred to the aquacultural facilities of the University of Helsinki and kept under

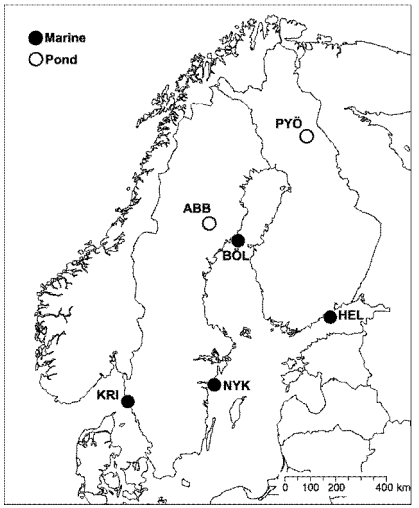


Fig. 1 Map of Scandinavia showing the nine-spined stickleback populations used in the study. KRI, Kristineberg; NYK, Nyköping; BÖL, Bölesviken; HEL, Helsinki; ABB, Abbortjärn; PYÖ, Pyöreälampi.

Correspondence: Gábor Herczeg, Ecological Genetics Research Unit, Department of Biosciences, PO Box 65, FI-00014, University of Helsinki, Helsinki, Finland.
Tel.: +358 9 191 57707; fax: +358 9 191 57694;
e-mail: gabor.herczeg@helsinki.fi

© 2011 THE AUTHORS. *J. EVOL. BIOL.* **24** (2011) 2434–2444
JOURNAL OF EVOLUTIONARY BIOLOGY © 2011 EUROPEAN SOCIETY FOR EVOLUTIONARY BIOLOGY

permanent light (natural summer condition at high latitudes) in 17 °C water and were fed with frozen bloodworms *ad libitum* until enough fish (for producing full-sib families) reached reproductive condition. Coastal marine sticklebacks are sympatric to a large number of predatory fish species, whereas in typical pond habitats, the nine-spined stickleback is the only fish species. According to our field experience, stickleback densities are considerably higher in ponds than in marine sites. We note that small-sized whitefish (*Coregonus lavaretus*) were recently introduced to one of the ponds (Pyöreälampi; Kalevi Kuusela, personal communication) in small numbers (we never caught a single whitefish besides thousands of sticklebacks), but based on their diet, whitefish are competitors rather than predators of sticklebacks (Kahilainen *et al.*, 2004). Predation by aquatic insects and insect larvae, and cannibalism might be present at all sites. Although ponds do not house permanent piscivorous birds due to their small surface area, the effect of sporadic visiting bird predators cannot be excluded. However, there are two facts that indicate relaxed predation and adaptation to this relaxed predation in the ponds: first, pond fish live ca. two times longer in nature than marine (or lake) fish (Herczeg *et al.*, 2009b), and second, pond nine-spined sticklebacks have undergone strong reduction in their defensive armour when compared to marine (or lake) nine-spined sticklebacks (Herczeg *et al.*, 2010a).

Six to ten artificial crosses per population (Abbortjärnen = 6, Bölesviken = 6, Helsinki = 8, Kristineberg = 10, Nyköping = 8, Pyöreälampi = 7) were made. For fish husbandry, we applied the methods outlined by Herczeg *et al.* (2009a) with minor modifications. In short, after hatching, individual fry (aiming for equal family/population/treatment representation) were placed randomly into individual 1.4-L containers on one of four Allentown Zebrafish Rack Systems (Aquaneering Inc., San Diego, CA, USA, hereafter 'rack') using physical, chemical, biological and UV filters. There were 100 containers per rack, with 400 individual containers in total. Visual communication between containers was blocked by white plastic plates. Individual rearing was important to avoid the confounding effects stemming from population divergence in mean aggression and the consequent population divergence in the costs of living in dense groups without the chance of leaving the group (Gonda *et al.*, 2009b; Herczeg *et al.*, 2009a,c). The photoperiod was set to 14 : 10 (light/dark), and the water temperature was held at 12 °C. Feeding was started with living brine shrimp (*Artemia salina*) nauplii and after 80 days gradually changed to frozen bloodworms. Mortality occurred mainly at the early life stages, and some fish managed to escape the system. In total, 315 fish was tested at the age of 30 weeks. Tested fish had a standard length (measured from the tip of the nose to the end of the tailbase) of ca. 3–5cm. After all the behavioural tests were completed, fish were killed with MS 222 (tricaine methanesulfonate), and their sex was identified by gonadal inspection. However, sex identification was not possible in some cases, and so the total number of individuals for analysis was 306 (Abbortjärnen = 37, Bölesviken = 46, Helsinki = 59, Kristineberg = 68, Nyköping = 50, Pyöreälampi = 46).

We applied a 2 × 2 factorial experiment with manipulated perceived predation risk and food supply. For the predation treatment, we fitted a 105-L external tank to every rack. Filtered water flowed into these tanks, and water was pumped from these tanks to the individual containers. Two 10- to 15-cm-long European perches (*Perca fluviatilis*) were placed into each of two randomly chosen external tanks ('predator present' treatment) to provide chemical stimuli from a common stickleback predator. There were no fish in the other two external tanks ('predator absent' treatment). The salinity of Baltic Sea is very low, especially at the coastal region, where European perch and nine-spined stickleback are both abundant. European perch is also one of the most abundant predatory fish in Fennoscandian freshwaters, including lakes, rivers and ponds. Hence, it was an ideal source of predatory cues for our experiment. Perch were fed with frozen bloodworms. For the food treatments, sticklebacks within family, predation treatment and rack were divided randomly into 'high food' (two feedings per day *ad libitum*) and 'low food' (one feeding per 2 days *ad libitum*) treatments. Our food treatments had a considerable effect on growth (K. Välimaki & G. Herczeg, unpublished). Hence, we assume we could effectively manipulate available energy.

Behavioural tests

We measured four behaviours during 8 weeks in spring 2010. We chose three of the five major personality trait categories (Réale *et al.*, 2007): risk-taking (= boldness), exploration and aggression. We also measured feeding activity under normal conditions, a behaviour that might be highly relevant in our nine-spine stickleback system including giant populations with distinct growth strategies (Herczeg *et al.*, 2009b, 2011). The largest age difference between families was 4 weeks; however, we tested every fish at the same age. Tests were carried out in the following order: feeding activity, risk-taking, exploration and aggression. Feeding activity and risk-taking were tested on fish that were 30–31 weeks old during separate morning feeding events. Exploration was tested in week 32 and aggression in week 33. Only the exploration test was performed out of the focal fish's home container, and fish were allowed to settle in their containers for at least 6 days prior to the aggression experiment. Exploration and aggression tests for low food treatment fish were performed on feeding days, so all fish were tested after being fed in the morning. All tests were performed by the same observer. At the end of the experiment, fish were over-anesthetized with MS 222 and photographed. Standard length was measured from the photographs using the software tpsDig 2.15 (Rohlf, 2006).

Feeding activity

Feeding activity was estimated as the time taken to initiate feeding in a normal feeding event. Each 1.4-L container had a feeding hole on the top, through which all food was given. Bloodworms were provided in excess through the hole with a pipette during a normal morning feeding event, and the time until the first biting attempt occurred was measured with a stopwatch. If a fish did not initiate feeding within 3 min, the experiment was terminated and the fish was assigned a time of 180 s.

Risk-taking

We measured risk-taking by following, e.g., Bell (2005): we assessed willingness to feed after a simulated attack. For this, a shiny screw (18 cm long, 1 cm diameter) was dropped through the feeding hole to the individual container of the focal fish. Considering the container's dimensions (15 × 5 × 25 cm, height, width, length, respectively, the shape was not completely regular), the sudden appearance of the screw together with its drop to the bottom of the container was envisioned to be perceived as a threat by the completely predator-naive focal fish. Bloodworms were provided immediately, and time until the first biting attempt was measured with a stopwatch. If a fish did not initiate feeding within 3 min, the experiment was terminated and the fish was assigned a time of 180 s.

Exploration

To estimate exploration, we applied a dark start box with a sliding door (18 cm × 10 cm × 10 cm, height, width and depth, respectively; for a similar approach see e.g. Brown *et al.*, 2007) combined with a simple maze (Fig. 2). The experimental tank (17 cm × 55 cm × 33 cm, height, length, and width, respectively) was filled up with water from the focal fish's rack reservoir to the depth of 10 cm. The focal fish was then gently netted out from its home container and placed into the starting box. We initially let the fish settle for 5 min. The sliding door was then opened with a string, and we used a stopwatch to measure the time until the focal fish (i) left the start box, (ii) crossed line 1 and (iii) crossed line 2 in the maze (see Fig. 2). If a fish did not leave the start box for 5 min, the experiment was terminated and the fish was assigned the time of 300 s for all measurements.

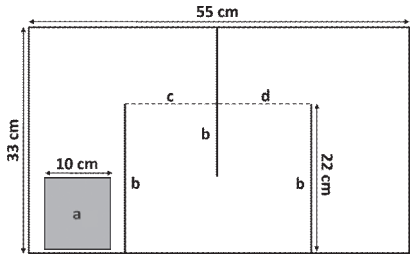


Fig. 2 Schematic representation of the maze used to estimate exploration. 'a' denotes the start box; 'b', the opaque dividers; 'c', the first line; and 'd', the second line to be crossed by the focal fish. The height of the tank was 17 cm.

Aggression

Due to the mortality that occurred at the beginning of the common garden experiment, there were over 80 empty containers in the experimental system. During week 28, the individual containers were reorganized so that the oldest focal fish had empty containers on both sides of their own container. As fish were removed from the racks after their testing finished, the number of empty containers increased during the testing period. Hence, all fish tested for aggression could have empty containers on both sides of their own container without any moving-related disturbance. As we kept extra fish from the families (pooled within population), we could present a new fish of the same age and population as stimulus to every focal fish.

A smaller stimulus fish was placed in a tank on a random side of the focal fish 2 days before the test to acclimate the stimulus fish to the setting. To start the experiment, the white plastic panels blocking vision at both sides of the focal fish's container were removed. This was important for separating aggression from the reaction to a novel object (i.e. we provided both a container with a stimulus fish inside and an empty container). After this, we waited for 3 min until the first orientation of the focal fish (being head-first towards the stimulus fish with the eyes fixed on it). If the first orientation happened within 3 min, we observed the focal fish's behaviour for another 3 min. During the observation period, we measured the time that the focal fish spent with orientation and the number of attacks (sudden swimming bursts often coupled with biting attempts) it made against the stimulus fish (Herczeg *et al.*, 2009a). If the focal fish did not orient towards the stimulus fish during the first 3 min, the experiment was terminated, and the focal fish was assigned zero time spent with orientation and zero number of attacks.

Statistical analyses

In the feeding activity test, 151 fish initiated feeding within 60 s, whereas 161 did not initiate feeding during the 3-min observation (only three fish ate after the first 60 s). In the risk-taking test, 137 fish initiated feeding within 60 s, whereas 169 fish did not eat during the 3-min observation (only nine fish ate after 60 s). Therefore, we simply created two categories, responders *vs.* nonresponders, for these behaviours. Note that leaving

out the small number of individuals eating after 60 s did not change our results qualitatively (data not shown), and thus we report the original analyses. We analysed these behaviours with generalized linear mixed models (GLMM) using PROC GLIMMIX as implemented in SAS 9.2 (SAS institute Inc., Cary, NC, USA) with a binomial error and logit link.

Aggression and exploration were represented by two and three measures, respectively; hence, we run principal component analyses (PCA) to obtain single variables describing the behaviours. In both cases, only the first PC had an eigenvector > 1 (PC aggression = 1.62; PC exploration = 2.32), the original variables loaded strongly and positively on the PCs (all loadings > 0.81), and the PCs described 80% (aggression) and 77% (exploration) of the original variation, respectively. PC aggression described a gradient from peaceful (low values) to aggressive (high values) fish, whereas PC exploration described a gradient from explorer (low values) towards sedentary (high values) fish. We used the PC scores as dependent variables in the subsequent multivariate general linear model (GLM) analysis. In this GLM, we first evaluated the multivariate results, and upon significant effects, we also considered the univariate tests.

Both the GLMMs and the multivariate GLM were built with habitat (marine *vs.* pond), sex, predation and food treatments as fixed factors, and standard length as a covariate. In the initial models, we included all two-way interactions. We also added population nested within habitat as a factor to control for the nonindependence of individuals within habitat type. Whenever populations within habitat differed significantly, we reran the same models with population instead of habitat as a fixed factor. Because we had a number of factor × covariate interactions in the models, we applied model selection (Engqvist, 2005). We performed a backward stepwise model selection based on the $P < 0.05$ criterion. We first removed the nonsignificant interactions in the order of decreasing P and then did the same with single effects. Single effects participating in significant interactions were never removed. We note that there are a number of different model selection methods available, but our method is considered as a conservative choice (Murtaugh, 2009).

Our sample size did not allow us to estimate family effects, so individuals were pooled within population. However, we believe that by using fish from 6 to 10 independent families from every population, we had a good representation of the populations' genetic variation. We did not apply statistical techniques (e.g. PCA) to obtain one or more independent variables describing all behaviours (behavioural type *sensu* Bell, 2007; see also Herczeg *et al.*, 2009a; David *et al.*, 2011). As our primary goal was to disentangle the effects of evolutionary history, ontogenetic experience and sex on the different behaviours, we analysed the four measured behaviours separately. All analyses were performed with SAS 9.2 (SAS institute Inc., Cary, NC, USA) and PASW 18 (SPSS Inc., Chicago, IL, USA) for Windows.

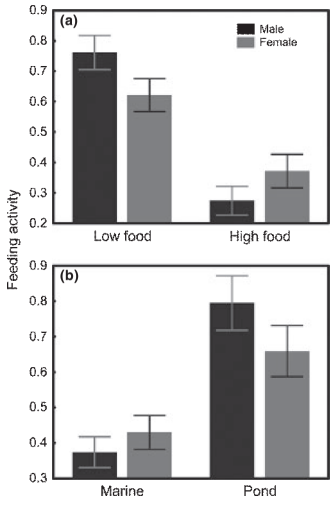


Fig. 3 (a) Sex-dependent food treatment and (b) sex-dependent habitat effects on feeding activity (initiating feeding or not in a normal situation; 1 = feeders, 0 = nonfeeders) in nine-spined sticklebacks. Means ± standard errors are provided.

Results

Feeding activity depended on food treatment, but this effect also interacted with sex (GLMM, sex: $F_{1,301} = 0.58$, $P = 0.45$; food: $F_{1,301} = 39.05$, $P < 0.001$; food × sex: $F_{1,301} = 4.31$, $P = 0.0039$; Fig. 3a). Poorly fed fish were quicker to start feeding in general, and males tended to initiate feeding more likely than females in the low food treatment, whereas females tended to start feeding more quickly than males in the high food treatment (Fig 3a). Habitats differed significantly, and the habitat × sex interaction also approached significance (habitat: $F_{1,301} = 23.55$, $P < 0.001$; habitat × sex: $F_{1,300} = 3.42$, $P = 0.065$; Fig. 3b). Pond fish had higher feeding activity than marine fish. Male pond sticklebacks were more likely to eat than pond females, whereas no such difference was evident in marine fish. The other effects in the model were nonsignificant (all $P > 0.19$; Appendix S1).

The second GLMM on risk-taking revealed a significant habitat-dependent food treatment effect (habitat: $F_{1,301} = 11.67$, $P < 0.001$; food: $F_{1,301} = 41.91$, $P < 0.001$; habitat × food: $F_{1,301} = 4.47$, $P = 0.035$, Fig. 4a). Although food shortage made all fish more likely to feed after a simulated attack, the effect was more pronounced in pond than in marine sticklebacks. Also, perceived predation risk influenced risk-taking ($F_{1,301} = 4.93$, $P = 0.027$, Fig. 4b): fish developing under perceived predation risk were less likely to initiate feeding under potential threat than those developing in control treatment. The other effects in the model were nonsignificant (all $P > 0.12$; Appendix S1).

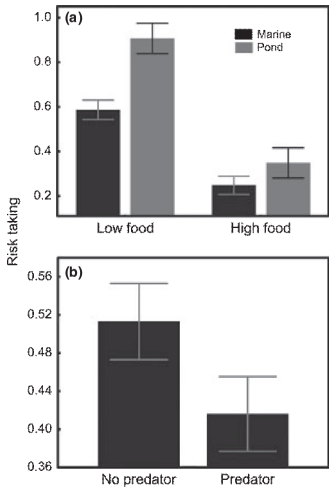


Fig. 4 (a) Habitat-dependent food treatment effect and (b) the effect of perceived predation risk on risk-taking (initiating feeding or not after a simulated attack; 1 = feeders, 0 = nonfeeders) in nine-spined sticklebacks. Means ± standard errors are provided.

The multivariate GLM revealed significant effects of habitat and predation treatments on exploration and aggression (habitat: Wilk's $\lambda_{2,296} = 0.81$, $P < 0.001$; predation: Wilk's $\lambda_{2,296} = 0.96$, $P = 0.003$). However, population nested within habitat was also significant (Wilk's $\lambda_{8,592} = 0.84$, $P < 0.001$). The other effects in the model were nonsignificant (all $P > 0.12$; Appendix S2). The population-level multivariate GLM revealed a significant effect of population and predation (population: Wilk's $\lambda_{10,592} = 0.69$, $P < 0.001$; predation: Wilk's $\lambda_{2,296} = 0.96$, $P = 0.003$). The other effects in the model were nonsignificant (all $P > 0.12$; Appendix S2). Subsequent univariate tests showed that population affected both behaviours (exploration: $F_{5,297} = 8.60$, $P < 0.001$; aggression: $F_{5,297} = 18.44$, $P < 0.001$). Fish from Pyöreälampi were more explorative than fish from the remaining populations (Fig. 5a). Pond sticklebacks were in general more aggressive than marine fish, with Pyöreälampi fish being more aggressive than Abbortjärnen fish (Fig. 5b). Predation treatment affected aggression ($F_{1,297} = 10.29$, $P = 0.001$) but not exploration ($F_{1,297} = 1.44$, $P = 0.23$). Fish under perceived predation risk were less aggressive than their conspecifics developing in the absence of predatory cues (Fig. 5c).

Discussion

The main aim of our study was to see how evolutionary history, ontogenetic experience, sex and their interactions affect behavioural variation within a single species. We studied feeding activity, risk-taking, aggression and exploration in F1 common garden nine-spined sticklebacks from high predation *vs.* high intraspecific competition populations reared under presence/absence of predatory cues and high/low food supply in a factorial design and not only found strong habitat, treatment and sex effects but also detected habitat- and sex-dependent treatment effects as well as habitat-dependent sex effects.

An interaction between evolutionary history and ontogenetic experience

We manipulated two environmental factors, which might have played an important role in the local adaptation of our populations, measured four behaviours and found one habitat × treatment interaction. Pond fish were more risk-taking, i.e. they were more likely than marine fish to initiate feeding after a simulated attack, but this was only the case when the fish had developed under food shortage. Results from experiments testing for habitat-dependent behavioural plasticity by manipulating the main components of the selective environment in laboratory are mixed. By testing how anuran tadpoles from populations with different predatory regimes alter their behaviour in response to predatory cues, both Laurila (2000) and Van Buskirk & Arioli (2005) reported strong behavioural plasticity expressed similarly in all populations. However, research on different fish species provided examples of population-dependent behavioural plasticity, usually populations being adapted to a certain environmental factor showing higher plasticity induced by the manipulation of that factor (Magurran, 1990; Rodd & Sokolowski, 1995; Salonen & Peuhkuri, 2007; for divergence within one lake see Robinson *et al.*, 2008). By studying Cladocerans, de Meester (1993, 1996) found that clones from natural populations with sympatric predatory fish are more responsive to predatory cues (increased negative phototactic behaviour) than clones from predator-free environments. In our case, predation-induced behavioural plasticity did not differ between populations or habitats, whereas the manipulation of available food induced a habitat-specific behavioural response. This pattern is understandable, considering

© 2011 THE AUTHORS. J. EVOL. BIOL. 24 (2011) 2434–2444
JOURNAL OF EVOLUTIONARY BIOLOGY © 2011 EUROPEAN SOCIETY FOR EVOLUTIONARY BIOLOGY

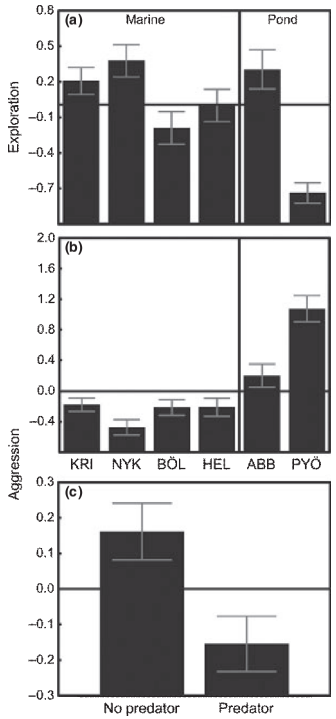


Fig. 5 Population divergence in (a) exploration (lower values represent more explorative fish), (b) aggression (higher values represent more aggressive fish) and (c) the effect of perceived predation risk on aggression in nine-spined sticklebacks. KRI, Kristineberg; NYK, Nyköping; BÖL, Bölesviken; HEL, Helsinki; ABB, Abbortjärn; PYÖ, Pyöreälampi. For a map of the sampled populations, see Fig. 1. Means ± standard errors are provided. The solid lines denote zero.

that less predictable environmental factors supposed to select for higher behavioural plasticity (Carroll & Corneli, 1999). In our system, the mortality risk imposed by piscine predators might be stable in both habitats, whereas the fluctuation in food availability might be higher in the isolated ponds more exposed to stochastic events (affecting both stickleback and prey densities) than in coastal marine habitats.

Biro *et al.* (2005) showed that young salmonids take higher risk under food shortage. The same effect was habitat dependent in our case. It has been shown that size-unlimited predation selects against both large body size and quick growth (e.g. Blanckenhorn, 2000; Biro *et al.*, 2004, 2006; for the opposite effect of size-limited predation, see e.g. Reimchen, 1991; Urban, 2008), whereas interspecific competition in a diverse community selects against any size shifts (Wilson, 1975; Lomolino, 1985; Simberloff *et al.*, 2000). Hence, when selective forces stemming from predation and interspecific competition are relaxed, increased growth and gigantism are expected to occur due to different forms of sexual selection all favouring large size (Sibly & Calow, 1986; Shine, 1989; Andersson, 1994; Blanckenhorn & Demont, 2004). This is indeed the situation in ponds, where the nine-spined stickleback is the only fish species, and gigantism (with a genetic basis) has been observed in different populations (Herczeg *et al.*, 2009b, 2011). Mathematical models support that large body size is critical for nine-spined sticklebacks' fitness in pond habitats (S. Aikio, G. Herczeg, A. Kuparinen & Juha Merilä, unpublished): as longevity is doubled in ponds (Herczeg *et al.*, 2009c) and nine-spine stickleback fecundity is size-dependent (Heins *et al.*, 2003, 2005; Herczeg *et al.*, 2010b), the fecundity advantage of large body size in ponds can outweigh the benefit of quick reproduction at small size. Hence, it is easy to understand why pond sticklebacks that develop under conditions with insufficient food supply take higher risks to feed than their marine conspecifics.

The effects of ontogenetic experience

Predation treatment affected risk-taking and aggression independent of habitat of origin and sex. Fish developing under perceived predation risk became more risk averse and less aggressive than sticklebacks developing in the absence of the chemical cues of a predator. We expected predation-adapted marine sticklebacks to show a larger shift in behaviour towards perceived predatory risk than pond sticklebacks, considering that both the maintenance of the capacity for plasticity and its expression are assumed to be costly (DeWitt, 1998; Auld *et al.*, 2010). However, we found no indication of habitat-dependent predation effect by manipulating the presence/absence of olfactory cues of European perch, a common stickleback predator of the Baltic Sea and freshwaters of Fennoscandia. It is difficult to predict how the behavioural plasticity in response to predators in populations with relaxed predation pressure should develop. Animals may retain their original ability to recognize predators or lose it slowly over time (Fong *et al.*, 1995). In some instances, behavioural responses to predation have been retained over very long periods (Coss, 1991; Blumstein & Daniel, 2002; Blumstein, 2006; Messler *et al.*, 2007; Lahti *et al.*, 2009). Anti-predatory responses might be retained when they are involved in some other important functions, connected to critical neurological processes, or maintained by complex gene interactions (Coss, 1999; Magurran, 1999; Blumstein & Daniel, 2002; Blumstein, 2006). At any rate, in our population system where predation-related adaptations have been reported in morphology, life history and behaviour (Gonda *et al.*, 2009a,b; Herczeg *et al.*, 2009a,b,c, 2010a, 2011), sticklebacks from both high- and low-predation habitats showed the same behavioural shifts in response to perceived predation risk, suggesting that they all have retained the ability to identify European perch as a predator. Food restriction increased feeding activity and risk-taking, however, the effects of food manipulation were sex- or habitat-dependent.

The effects of evolutionary history

The behaviour of nine-spined sticklebacks differed markedly among habitats. Pond sticklebacks were more likely to initiate feeding and to take risk and were more aggressive and explorative than marine sticklebacks. Intraspecific geographical variation in behaviour is common (e.g. Foster & Endler, 1999), and there are numerous examples of covariation between behaviour and predation risk across populations (e.g. Magurran & Seghers, 1991, 1994; Brown *et al.*, 2005; Álvarez & Bell, 2007; Herczeg *et al.*, 2009a). The fact that the patterns we report are likely to have a genetic basis and that they were habitat dependent implies that natural selection is the causal agent (e.g. Clarke, 1975; Endler, 1986; McGuigan *et al.*, 2005). We note that the number of replicates for the pond habitat was low, but the two ponds were separated by more than 500 km and are known to be genetically highly isolated (Shikano *et al.*, 2010), and thus truly independent. We also found significant population variation in aggression and exploration within habitat type. Both pond populations were more aggressive than the marine populations, with Pyöreälampi fish being more aggressive that Abbortjärn fish. Pyöreälampi fish were more explorative than the other populations, Abbortjärnen fish being indistinguishable from marine fish. It seems that behaviour evolved in similar directions in the two completely independent ponds, but Abbortjärnen fish diverged less from the marine conspecifics than Pyöreälampi fish.

Interestingly, animal behaviour can adapt to high predation risk in two opposite ways. One might intuitively expect prey to evolve to be shyer and less active when exposed to predators (e.g. Bell, 2005; Brydges *et al.*, 2008). However, Brown *et al.* (2005, 2007) reported the opposite pattern: individuals of the poeciliid fish *Brachyraphis episcope* showed higher activity and were bolder (also in laboratory generations) under higher predation pressure. The authors interpreted this pattern as being due to fish under high predation risk needing to be bolder in order to carry on with necessary activities such as feeding and finding mates (Brown *et al.*, 2005, 2007). According to our results, nine-spined sticklebacks adapt to high predation by becoming more risk averse and less aggressive. These results are in agreement with results from our preliminary study using partly different populations (Herczeg *et al.*, 2009a), suggesting that the pattern is general in the species.

Sex effects

We also found that sex affected feeding activity. Males were more likely to initiate feeding than females in the low food treatment, whereas the opposite was found in the high food treatment. Further, the habitat × sex interaction also approached significance ($P = 0.065$): pond males were more likely to initiate feeding than pond females, whereas no such effects were observed in marine sticklebacks. These patterns are not straightforward to interpret. The behaviour of male and female sticklebacks is strikingly different during reproduction (e.g. Wooton, 1976; Bell & Foster, 1994). However, by setting the photoperiod and temperature to mimic nonreproductive periods, we avoided fish reaching reproductive condition in our experiment, and so the different behavioural roles and energy requirements during reproduction are unlikely to explain the patterns directly. This suggests that the differences might be due to sex-dependent life history strategies. It has been shown that females are larger than males, especially in ponds (Herczeg *et al.*, 2010b), and that the growth strategy differences between habitats are also driven by females (Herczeg *et al.*, 2011). Based on this, one might expect females to have a higher feeding activity. However, the only situation where this trend occurred was in the high food treatment – in the low food treatment and in pond populations in general, males were more likely to feed.

Conclusion

In summary, we found that evolutionary history, ontogenetic experience and sex all affected the behaviour of completely predator- and conspecific-naive, laboratory-reared nine-spined sticklebacks. Pond fish were more likely to feed, took more risk and were more aggressive and also more explorative than marine fish. Exposure to the chemical cues of a predator during development made all fish less risk-taking and less aggressive. Food shortage made sticklebacks more likely to initiate feeding both in 'normal' and potentially threatening situations. Although we found sexual differences too, they were context dependent and hard to interpret. Perhaps, the most salient finding of the present study was the habitat-dependent expression of behavioural plasticity in one particular case: pond fish were more risk-taking than marine fish, but only when they had developed under food shortage. As the patterns reported here are likely to have genetic basis, we suggest that the habitat-dependent behavioural plasticity results from natural selection. Our study

© 2011 THE AUTHORS. *J. EVOL. BIOL.* **24** (2011) 2434–2444
JOURNAL OF EVOLUTIONARY BIOLOGY © 2011 EUROPEAN SOCIETY FOR EVOLUTIONARY BIOLOGY

demonstrates how complex interactions between genetic and environmental factors shape different behaviours in different ways.

Acknowledgments

Pirkko Siikamäki, Riku Paavola, Pia Rännänen, Göran Englund, Jenni Hämäläinen, Jacquelin DeFaveri and Tuomas Leinonen helped to obtain the parental fish. We are highly indebted to the Oulanka Research Station (University of Oulu) for help with the fieldwork. Mirva Turtiainen, Linda Uoti, Maarit Haataja, Nurul Izza Ab Ghani, Riikka Kaartinen, Joona Lehtomäki, Yukinori Shimada and Marika Lilja helped with the experiment. We thank Amber Teacher for correcting the English. Our work was funded by the Academy of Finland (GH; JM) and the Finnish Graduate School in Population Genetics (KV). The experiment was conducted under a licence from the Finnish National Animal Experiment Board (Licence number STH223A).

References

Äbjörnsson, K., Hansson, L.-A. & Brönmark, C. 2004. Responses of prey from habitats with different predator regimes: local adaptation and heritability. *Ecology* **85**: 1859–1866.

Álvarez, D. & Bell, A. 2007. Sticklebacks from streams are more bold than sticklebacks from ponds. *Behav. Proc* **76**: 215–217.

Andersson, M. 1994. *Sexual Selection*. Princeton University Press, Princeton.

Auld, J.R., Agrawal, A.A. & Relyea, R.A. 2010. Re-evaluating the costs and limits of adaptive phenotypic plasticity. *Proc. R. Soc. B* **277**: 503–511.

Bănărescu, P.M. & Paepke, H.-J. 2001. *The Freshwater Fishes of Europe*, Vol. **5/III**. AULA-Verlag, Wiebelsheim.

Bell, A.M. 2005. Behavioural differences between individuals and two populations of stickleback (*Gasterosteus aculeatus*). *J. Evol. Biol.* **18**: 464–473.

Bell, A.M. 2007. Future directions in behavioural syndrome research. *Proc. R. Soc. B* **274**: 755–761.

Bell, M.A. & Foster, S.A. 1994. *The Evolutionary Biology of Threespine Stickleback*. Oxford University Press, Oxford.

Biro, P.A., Abrahams, M.V., Post, J.R. & Parkinson, E.A. 2004. Predators select against high growth rates and risk-taking behaviour in domestic trout populations. *Proc. R. Soc. Lond. B* **271**: 2233–2237.

Biro, P.A., Post, J.R. & Abrahams, M.V. 2005. Ontogeny of energy allocation reveals selective pressure promoting risk-taking behaviour in young fish cohorts. *Proc. R. Soc. B.* **272**: 1443–1448.

Biro, P.A., Abrahams, M.V., Post, J.R. & Parkinson, E.A. 2006. Behavioural trade-offs between growth and mortality explain evolution of submaximal growth rates. *J. Anim. Ecol.* **75**: 1165–1171.

Blanckenhorn, W.U. 2000. The evolution of body size: what keeps organisms small? *Q. Rev. Biol.* **75**: 385–407.

Blanckenhorn, W.U. & Demont, M. 2004. Bergmann and converse Bergmann latitudinal clines in arthropods: two ends of a continuum? *Integr. Comp. Biol.* **44**: 413–424.

Blumstein, D.T. 2006. The multi-predator hypothesis and the evolutionary persistence of antipredator behavior. *Ethology* **112**: 209–217.

Blumstein, D.T. & Daniel, J.D. 2002. Foraging behavior of three Tasmanian macropodid marsupials in response to present and historical predation threat. *Ecography* **26**: 585–594.

Breden, F., Scott, M.A. & Michel, E. 1987. Genetic differentiation for anti-predator behaviour in the Trinidad guppy, *Poecilia reticulata*. *Anim. Behav.* **35**: 618–620.

Brown, C., Jones, F. & Braithwaite, V.A. 2005. *In situ* examination of boldness-shyness traits in the tropical poeciliid, *Brachyraphis episcope*. *Anim. Behav.* **70**: 1003–1009.

Brown, C., Burgess, F. & Braithwaite, V.A. 2007. Heritable and experiential effects on boldness in a tropical poeciliid. *Behav. Ecol. Sociobiol.* **62**: 237–243.

Brydges, N.M., Colegrave, N., Heathcote, R.J.P. & Braithwaite, V.A. 2008. Habitat stability and predation pressure affect temperament behaviours in populations of three-spined sticklebacks. *J. Anim. Ecol.* **77**: 229–235.

Carroll, S.P. & Corneli, P.S. 1999. The evolution of behavioral norms of reaction as a problem in ecological genetics. In: *Geographic Variation in Behavior: Perspectives on Evolutionary Mechanisms* (S.A. Foster & J.A. Endler, eds), pp. 52–68. Oxford University Press, Oxford.

Clarke, B.C. 1975. The contribution of ecological genetics to evolutionary theory: detecting the direct effects of natural selection on particularly polymorphic loci. *Genetics* **79**(Suppl.): 911–922.

Coss, R.G. 1991. Evolutionary persistence of memory-like processes. *Concepts Neurosci.* **2**: 129–168.

Coss, R.G. 1999. Effects of relaxed natural selection on the evolution of behavior. In: *Geographic Variation in Behavior: Perspectives on Evolutionary Mechanisms* (S.A. Foster & J.A. Endler, eds), pp. 180–208. Oxford University Press, Oxford.

Crispo, E. 2007. The Baldwin effect and genetic assimilation: revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. *Evolution* **61**: 2469–2479.

Crispo, E. 2008. Modifying effects of phenotypic plasticity on interactions among natural selection, adaptation and gene flow. *J. Evol. Biol.* **21**: 1460–1469.

David, M., Auclair, Y. & Cézilly, F. 2011. Personality predicts social dominance in female zebra finches, *Taeniopygia guttata*, in a feeding context. *Anim. Behav.* **81**: 219–224.

DeWitt, T. 1998. Costs and limits of phenotypic plasticity. *Trends Ecol. Evol.* **13**: 77–81.

Dingemanse, N.J., Van der Plas, F., Wright, J., Réale, D., Schrama, M., Roff, D.A. *et al.* 2009. Individual experience and evolutionary history of predation affect expression of heritable variation in fish personality and morphology. *Proc. R. Soc. B* **276**: 1285–1293.

Endler, J. 1977. *Geographic Variation, Speciation and Clines*. Princeton University Press, Princeton.

Endler, J.A. 1986. *Natural Selection in the Wild*. Princeton University Press, Princeton.

Engqvist, L. 2005. The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Anim. Behav.* **70**: 967–971.

Fong, D.W., Kane, T.C. & Culver, D.C. 1995. Vestigialization and loss of nonfunctional characters. *Ann. Rev. Ecol. Syst.* **26**: 249–268.

Foster, S.A. 1999. The geography of behaviour: an evolutionary perspective. *Trends Ecol. Evol.* **14**: 190–195.

Foster, S.A. & Endler, J.A. 1999. *Geographic Variation in Behavior: Perspectives on Evolutionary Mechanisms*. Oxford University Press, Oxford.

Fusco, G. & Minelli, M. 2010. Phenotypic plasticity in development and evolution: facts and concepts. *Phil. Trans. R. Soc. B* **365**: 547–556.

Gabor, C.R. & Ryan, M.J. 2001. Geographical variation in reproductive character displacement in mate choice by male sailfin mollies. *Proc. R. Soc. Lond. B* **268**: 1063–1070.

Giles, N. & Huntingford, F.A. 1984. Predation risk and inter-population variation in anti-predator behaviour in the three-spined stickleback, *Gasterosteus aculeatus* L. *Anim. Behav.* **32**: 264–275.

Gonda, A., Herczeg, G. & Merilä, J. 2009a. Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? *J. Evol. Biol.* **22**: 1721–1726.

Gonda, A., Herczeg, G. & Merilä, J. 2009b. Habitat-dependent and -independent plastic responses to social environment in the ninespine stickleback (*Pungitius pungitius*) brain. *Proc. R. Soc. B* **276**: 2085–2092.

Heins, D.C., Johnson, J.M. & Baker, J.A. 2003. Reproductive ecology of the nine-spined stickleback from south-central Alaska. *J. Fish Biol.* **63**: 1131–1143.

Heins, D.C., Baker, J.A., De Silva, G. & Birden, E.L. 2005. Clutch characteristics of two populations of nine-spined stickleback from south-central Alaska. *J. Fish Biol.* **67**: 873–878.

Herczeg, G., Gonda, A. & Merilä, J. 2009a. Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *J. Evol. Biol.* **22**: 544–552.

Herczeg, G., Gonda, A. & Merilä, J. 2009b. Evolution of gigantism in nine-spined sticklebacks. *Evolution* **63**: 3190–3200.

Herczeg, G., Gonda, A. & Merilä, J. 2009c. The social cost of shoaling covaries with predation risk in nine-spined stickleback (*Pungitius pungitius*) populations. *Anim. Behav.* **77**: 575–580.

Herczeg, G., Turtiainen, M. & Merilä, J. 2010a. Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. *Biol. J. Linn. Soc.* **101**: 403–416.

Herczeg, G., Gonda, A. & Merilä, J. 2010b. Rensch's rule inverted – female-driven gigantism in nine-spined stickleback *Pungitius pungitius*. *J. Anim. Ecol.* **79**: 581–588.

Herczeg, G., Gonda, A., Kuparinen, A. & Merilä, J. 2011. Contrasting growth strategies of pond versus marine populations of nine-spined sticklebacks (*Pungitius pungitius*): a combined effect of predation and competition? *Evol. Ecol.* doi: 10.1007/s10682-011-9491-9.

Huntingford, F.A., Wright, P.J. & Tierney, J.F. 1994. Adaptive variation in antipredator behaviour in threespine stickleback. In: *The Evolutionary Biology of Threespine Stickleback* (M.A. Bell & S.A. Foster, eds), pp. 277–296. Oxford University Press, Oxford.

Kahilainen, K., Malinen, T., Tuomaala, A. & Lehtonen, H. 2004. Diel and seasonal habitat and food segregation of three sympatric *Coregonus lavaretus* forms in a subarctic lake. *J. Fish Biol.* **64**: 418–434.

Kawecki, T.J. & Ebert, D. 2004. Conceptual issues in local adaptation. *Ecol. Lett.* **7**: 1225–1241.

Lahti, D.C., Johnson, N.A., Ajie, B.C., Otto, S.P., Hendry, A.P., Blumstein, D.T. *et al.* 2009. Relaxed selection in the wild. *Trends Ecol. Evol.* **24**: 487–496.

Laurila, A. 2000. Behavioural responses to predator chemical cues and local variation in antipredator performance in *Rana temporaria* tadpoles. *Oikos* **88**: 159–168.

Lomolino, M.V. 1985. Body size of mammals on islands: the island rule re-examined. *Am. Nat.* **125**: 310–316.

Magurran, A.E. 1990. The inheritance and development of minnow anti-predator behaviour. *Anim. Behav.* **39**: 834–842.

Magurran, A.E. 1999. The diversification and development of antipredator behaviour: perspectives from fish populations. In: *Geographic Diversification of Behavior: an Evolutionary Perspective* (S.A. Foster & J.A. Endler, eds), pp. 139–163. Oxford University Press, Oxford.

Magurran, A.E. & Seghers, B.H. 1991. Variation in schooling and aggression amongst guppy (*Poecilia reticulata*) populations in Trinidad. *Behaviour* **118**: 214–234.

Magurran, A.E. & Seghers, B.H. 1994. Predator inspection behaviour covaries with schooling tendency amongst wild guppy, *Poecilia reticulata*. *Behaviour* **128**: 121–134.

Mathis, A., Chivers, D.P. & Smith, R.J.F. 1993. Population differences in responses of fathead minnows (*Pimephales promelas*) to visual and chemical stimuli from predators. *Ethology* **93**: 31–40.

Mayr, E. 1963. *Animal Species and Evolution*. Harvard University Press, Cambridge.

McGuigan, K., Chenoweth, S.F. & Blows, M.W. 2005. Phenotypic divergence along lines of genetic variance. *Am. Nat.* **165**: 32–43.

de Meester, L. 1993. Fish-mediated chemical, and phototactic behaviour in *Daphnia magna*. *Ecology* **74**: 1467–1474.

de Meester, L. 1996. Evolutionary potential and local genetic differentiation in a phonetically plastic trait of a cyclical parthenogen, *Daphnia magna*. *Evolution* **50**: 1293–1298.

Messler, A., Wund, M., Baker, J. & Foster, S. 2007. The effects of relaxed and reversed selection by predators on the antipredator behavior of the threespine stickleback, *Gasterosteus aculeatus*. *Ethology* **11**: 953–963.

Murtaugh, P.A. 2009. Performance of several variable-selection methods applied to real ecological data. *Ecol. Lett.* **12**: 1061–1068.

Östlund-Nilsson, S., Mayer, I. & Huntingford, F.A. 2007. *Biology of the Three-spined Stickleback*. CRC Press, Boca Raton.

Pfennig, D.W., Wund, M.A., Snell-Rood, E.C., Cruickshank, T., Schlichting, C.D. & Moczek, A.P. 2010. Phenotypic plasticity's impact on diversification and speciation. *Trends Ecol. Evol.* **25**: 459–467.

Pigliucci, M., Murren, C.J. & Schlichting, C.D. 2006. Phenotypic plasticity and evolution by genetic assimilation. *J. Exp. Biol.* **209**: 2362–2367.

Réale, D., Reader, S.M., Sol, D., McDougall, P.T. & Dingemanse, N.J. 2007. Integrating animal temperament within ecology and evolution. *Biol. Rev.* **82**: 291–318.

Reimchen, T.E. 1991. Trout foraging failure and the evolution of body size in stickleback. *Copeia* **1991**: 1098–1104.

Relyea, R.A. 2001. Morphological and behavioral plasticity of larval anurans in response to different predators. *Ecology* **82**: 523–540.

Riechert, S.E. & Hedrick, A.V. 1990. Levels of predation and genetically based antipredator behaviour in the spider, *Agelenopsis aperta*. *Anim. Behav.* **40**: 679–687.

Robinson, B.W., Januszkiewicz, A.J. & Koblitz, J.C. 2008. Survival benefits and divergence of predator-induced behav-

iour between pumpkinseed sunfish ecomorphs. *Behav. Ecol.* **19**: 263–271.
Rodd, F.H. & Sokolowski, M.B. 1995. Complex origins of variation in the sexual behaviour of male Trinidadian guppies, *Poecilia reticulata*: interactions between social environment, heredity, body size and age. *Anim. Behav.* **49**: 1139–1159.
Rohlf, F.J. 2006. *TPS Software Series.* Distributed by the author. Stony Brook, NY: Department of Ecology and Evolution, State University of New York.
Salonen, A. & Peuhkuri, N. 2007. Aggression level in different water velocities depends on population origin in grayling, *Thymallus thymallus*. *Ethology* **113**: 39–45.
Shaw, K.A., Scotti, M.L. & Foster, S.A. 2007. Ancestral plasticity and the evolutionary diversification of courtship behaviour in threespine sticklebacks. *Anim. Behav.* **73**: 415–422.
Shikano, T., Shimada, Y., Herczeg, G. & Merilä, J. 2010. History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. *Mol. Ecol.* **19**: 1147–1161.
Shine, R. 1989. Ecological causes for the evolution of sexual size dimorphism: a review of the evidence. *Q. Rev. Biol.* **64**: 419–461.
Sibly, R. & Calow, P. 1986. Why breeding earlier is always worthwhile. *J. Theor. Biol.* **124**: 311–319.
Simberloff, D., Dayan, T., Jones, C. & Ogura, G. 2000. Character displacement and release in the small Indian mongoose, *Herpestes javanicus*. *Ecology* **81**: 2086–2099.
Steiner, U.K. 2007. Investment in defense and cost of predator-induced defense along a resource gradient. *Oecologia* **152**: 201–210.
Storfer, A. & Sih, A. 1998. Gene flow and ineffective anti-predatory behavior in a stream-breeding salamander. *Evolution* **52**: 558–565.
Tollerian, R. & Harvell, C.D. 1999. *The Ecology and Evolution of Inducible Defenses*. Princeton University Press, Princeton.
Urban, M.C. 2008. Salamander evolution across a latitudinal cline in gape-limited predation risk. *Oikos* **117**: 1037–1049.
Van Buskirk, J. & Arioli, M. 2005. Habitat specialization and adaptive phenotypic divergence of anuran populations. *J. Evol. Biol.* **18**: 596–608.
West-Eberhard, M.J. 1989. Phenotypic plasticity and the origins of diversity. *Ann. Rev. Ecol. Syst.* **20**: 249–278.
West-Eberhard, M.J. 2003. *Developmental Plasticity and Evolution*. Oxford University Press, Oxford.
Wilson, D.S. 1975. The adequacy of body size as a niche difference. *Am. Nat.* **109**: 769–784.
Wooton, R.J. 1976. *The Biology of Sticklebacks.* Academic Press, London.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Results from the Generalised Linear Mixed Models with binomial error and logit link ran on feeding activity (a) and risk-taking (b).

Appendix S2 Results from the multivariate General Linear Models on exploration and aggression, ran on the habitat (a) and on the population (b) level.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be reorganized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

Received 31 May 2011; revised 18 July 2011; accepted 19 July 2011

doi:10.1111/j.1558-5646.2009.00781.x

EVOLUTION OF GIGANTISM IN NINE-SPINED STICKLEBACKS

Gábor Herczeg,[1,2] Abigél Gonda,[1,3] and Juha Merilä[1,4]

[1]*Ecological Genetics Research Unit, Department of Biological and Environmental Sciences, University of Helsinki, PO Box 65, FI-00014, Helsinki, Finland*

[2]*E-mail: gabor.herczeg@helsinki.fi*

[3]*E-mail: abigel.gonda@helsinki.fi*

[4]*E-mail: juha.merila@helsinki.fi*

Received January 15, 2009
Accepted June 16, 2009

The relaxation of predation and interspecific competition are hypothesized to allow evolution toward "optimal" body size in island environments, resulting in the gigantism of small organisms. We tested this hypothesis by studying a small teleost (nine-spined stickleback, *Pungitius pungitius*) from four marine and five lake (diverse fish community) and nine pond (impoverished fish community) populations. In line with theory, pond fish tended to be larger than their marine or lake conspecifics, sometimes reaching giant sizes. In two geographically independent cases when predatory fish had been introduced into ponds, fish were smaller than those in nearby ponds lacking predators. Pond fish were also smaller when found in sympatry with three-spined stickleback (*Gasterosteus aculeatus*) than those in ponds lacking competitors. Size-at-age analyses demonstrated that larger size in ponds was achieved by both increased growth rates and extended longevity of pond fish. Results from a common garden experiment indicate that the growth differences had a genetic basis: pond fish developed two to three times higher body mass than marine fish during 36 weeks of growth under similar conditions. Hence, reduced risk of predation and interspecific competition appear to be chief forces driving insular body size evolution toward gigantism.

KEY WORDS: **Body size, competition, island rule, natural selection, predation, *Pungitius*.**

Individual and population level variation in body size often correlates with the variation in many physiological and fitness traits (Peters 1983; Roff 1992; Stearns 1992), making body size a trait of fundamental ecological and evolutionary importance and interest. In general, fecundity selection, male–male competition, and female mate choice often favor larger body size (Wooton 1979; Clutton-Brock et al. 1982; Shine 1988, 1989; Andersson 1994). Still, in many cases, evolution toward larger body size is not taking place, and the question "What keeps organisms small?" is highly relevant (Stanley 1973; Blanckenhorn 2000). Because predation is one of the main factors behind viability selection against gigantism, it can be a critical factor keeping organisms "small" (Blanckenhorn 2000). Likewise, by constraining individuals' possibilities for utilizing critical resources, interspecific competition can counteract selection favoring large body size (Wilson 1975; Lomolino 1985; Simberloff et al. 2000). Several other potential sources of interpopulation variation in body size have been proposed: random events (Wasserzug et al. 1979), different resource levels (Case 1978, 1979), prey size (Gittleman 1985; Forsman 1991; Boback 2003), and intraspecific interactions (Clegg and Owens 2002; Wu et al. 2006) can all significantly contribute to the body size variation among populations. Hence, in general, body size at the population level is a result of several co-occurring evolutionary and ecological forces that can be hard to disentangle.

Insular evolution has attracted considerable attention from ecologists as well as evolutionary biologists (MacArthur and Wilson 1967; Grant 1998), one reason being that island ecosystems are often less complex, with simpler selective pressures than mainland ones (Whittaker 1998; Schluter 2001). Studying evolutionary changes of a mainland organism following its establishment on islands is a powerful tool in the study of evolution; mainland–island systems can be viewed as natural experiments (e.g., Mayr 1967; Losos et al. 1998; Losos and Schluter 2000). Key features characterizing organisms in island habitats, as compared to mainland ones, are thought to be loss of dispersability (Whittaker 1998) and a relaxation of evolutionary forces stemming from predation pressure and interspecific competition (e.g., McNab 1994; Lomolino 2005).

Body size is among the traits that can change drastically as a response to insular environments, often being the only trait in which differences are ubiquitous (Case 1978). In the first comprehensive review of insular body size evolution of mammals, Foster (1964) pointed out that while some taxa tend to dwarf, others become giants on islands—a phenomenon coined the "island rule" by Van Valen (1973a,b). By revisiting the question, Lomolino (1985) supported Foster's (1964) finding that small-sized mammals tend to become giants, while larger species dwarf on islands. A popular and potential explanation for this pattern is that when a population is released from interspecific competition and predation, it evolves toward an intermediate "optimal" body size (Damuth 1993; Boback and Guyer 2003), which, however, might not represent a single value for large and diverse taxa like mammals (Meiri et al. 2005a). The island rule, originally formulated for mammals, has been widened to other taxa as well, earning support for vertebrates in general (e.g., Clegg and Owens 2002; Lomolino 2005). Parallel to the studies supporting the island rule, others arrived at opposite conclusions and rejected it (Lawlor 1982; Meiri et al. 2004, 2005b, 2006, 2008). Even inverse patterns, i.e., small species becoming smaller while large ones larger on islands, have been found among carnivorous lizards (Meiri 2007). Recent studies highlight the methodological challenges as well as the importance of proper null hypotheses and analytical tools for reliably supporting or rejecting the island rule (Lomolino et al. 2006; Meiri et al. 2008; Welch 2009).

However, the mechanism behind the insular body size evolution within species is also unclear. For instance, the possibility that the selective forces act only indirectly on body size by affecting life history traits is conceivable (Palkovacs 2003). Even though the ecogeographical rules were originally formulated in an interspecific context, intraspecific studies testing the given rule's predictions have also increased understanding of the mechanisms behind the large-scale patterns (see, e.g., Heaney 1978; Fairbairn and Preziosi 1994). In general, it seems that intraspecific studies, without bias from interspecific differences in physiological constraints and phylogenetic past, and with adequate knowledge about the target species' biology and the studied environments, offer the possibility for in-depth analysis and interpretation of insular body size shifts (e.g., Palmer 2002; Keogh et al. 2005; Wu et al. 2006). Surprisingly, apart from McClain et al.'s (2006) study which compared coastal (treated as mainland) versus deep sea (treated as island) gastropods, all studies on insular body size evolution have focused on terrestrial organisms. However, the "attractiveness" of islands for evolutionary biologists, namely the decreased complexity and simplified selective forces, can be much more pronounced in small isolated water bodies than on terrestrial islands. First, reduced gene flow in the case of obligatory water organisms (e.g., fish) can be complete in isolated water bodies. This is very important given gene flow's power to counteract natural selection (e.g., Hendry et al. 2002; Moore et al. 2007). Second, a complete release from interspecific interactions (competition and predation) is possible in the simple fish communities characterizing some small isolated ponds.

The aim of this study was to test the hypothesis that evolution toward large size in small fish is found in island habitats (=isolated ponds) where the selective forces—stemming from predation and interspecific competition—acting against large body size are relaxed. We did this by comparing the variation in the mean body size among Fennoscandian nine-spined stickleback (*Pungitius pungitius*) populations. This species represents an excellent model for this purpose, because it is found in a variety of different habitats from seas, lakes, and rivers to small creeks and ditches, successfully persisting also in isolated ponds, often as the only fish species (Bănărescu and Paepke 2001; Östlund-Nilsson et al. 2007). By comparing geographically distinct marine, lake, and pond populations (Fig. 1), we tested the following predictions. First, *P. pungitius* should be larger in ponds where predator or competitor fish species are absent than in other habitats housing complex fish faunas. Second, the presumed body size differences in the wild should result from the variation in growth rates, not only in lifespan. Third, the presumed differences in growth rate among populations should have a genetic (evolutionary) basis. In addition, we investigated the effect of introduction of predatory fish on the mean body size of the sticklebacks in habitats that formerly lacked predators.

Methods

SAMPLING SITES AND SAMPLE COLLECTION

Adult fish were collected from 16 Fennoscandian populations including four marine sites, five nonisolated large lakes (hereafter: lake), and seven isolated ponds (hereafter: pond; Fig. 1, Table 1) between 2003 and 2007. We define lakes as water bodies larger than 30 ha that are connected to creeks and rivers and thus, the stickleback populations in them are not isolated. On the other hand, ponds are smaller than 5 ha and completely isolated (both in- and outlets [when present] are small and steep), meaning no immigration possibilities at all. The sampled populations represent markedly different selective environments. Both

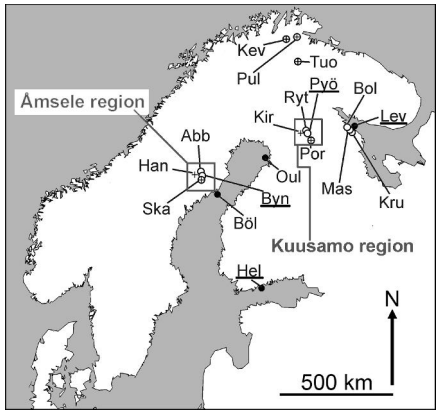


Figure 1. Map of Fennoscandia showing the location of the sampling sites. Open circles denote small isolated ponds without predatory fish, plus signs denote ponds with introduced *Salmo trutta*, while crossed circles denote large lake, and filled circles marine populations. For the population abbreviations see Table 1. The underlined populations are the ones represented in the common garden experiment. The dark gray boxes show the regions used in the analyses of predator introductions.

marine and lake populations are members of a diverse fish fauna, including several predatory (e.g., pike, *Esox lucius*, perch, *Perca fluviatilis*, different salmonids, for example brown trout, *Salmo trutta*) and numerous competitor fish species (e.g., juveniles of almost all sympatric fish). In contrast, in the isolated ponds there are no predatory fish at all, and due to the relatively small surface area, they do not house permanent fishing birds either. Predation by aquatic insects and possible cannibalism (relevant in early life stages) might be present in all sites. Two facts demonstrate the relaxed predatory pressure in the ponds: first, *P. pungitius* have undergone strong reduction in defensive armor (reduction or loss of pelvic apparatus) in all studied ponds (G. Herczeg, M. Turtiainen, J. Merila, unpubl. data) and second, fish in the ponds live longer than those in other populations (see the Results section). In the three Russian ponds (Bolotnoje, Krugloje, Mashinnoje), *P. pungitius* is sympatric with large three-spined sticklebacks (*Gasterosteus aculeatus*) that heavily outnumber *P. pungitius*. In one of the Finnish ponds (Pyöreälampi), a few small whitefish (*Coregonus lavaretus*; still larger than sticklebacks) have been introduced in the last century. However, due to their dietary habits (feeding on invertebrates; see, e.g., Kahilainen et al. 2004), they can be only considered as competitors. Further, due to their low number (we did not catch a single individual among the thousands of sticklebacks), even this competitor effect might be minimal. We included two additional ponds (Kirkasvetinenlampi, Finland; Hansmyrtjärn, Sweden; Fig. 1) where a common fish predator, *S. trutta*, has been introduced in the last century (exact date unknown) in one analysis.

The Baltic Sea (Helsinki, Oulu, Bölesviken) and the Russian samples (Levin Navolok, Bolotnoje, Krugloje, Mashinnoje) form two groups within which the populations are genetically indistinguishable, but these groups were found to be genetically isolated from each other and all the other studied populations (which were also highly isolated from each other in all possible combinations) based on highly variable microsatellite markers (Shikano et al. unpubl. data). The reason for the lack of genetic isolation within the Baltic and Russian groups is easy to understand: there is no barrier against the gene flow in the Baltic Sea despite the large geographic distance between the sample sites, while the Russian ponds became isolated only recently (within a century) from the White Sea (Ziuganov and Zotin 1995; information from the White Sea Biological Station).

In most cases, adult fish were collected in early summer (between 2003 and 2007), during the reproductive season (late May to early July) with the aid of seine nets and minnow traps. *P. pungitius* normally reaches maturity after its first wintering, and most of its growth is found before the first breeding season (Jones and Hynes 1950; Bănărescu and Paepke 2001), so distinguishing between adults and juveniles is very simple during the reproductive season. One lake, Tuolpujärvi, was sampled in early September. However, considering the general growth patterns (see above) and that data from Tuolpujärvi fitted with the patterns found (see the Results section), we did not exclude this population (exclusion did not alter our results qualitatively). It is noteworthy that we could not identify sex in some populations. However, analysis of a smaller dataset revealed that sexual size dimorphism is only relevant in the giant pond populations (G. Herczeg, A. Gonda, J. Merila, unpubl. data) and because they (1) represent one extreme of the size distribution and (2) had nearly equal sex representation in our sample, we did not consider sex differences in the analyses. The interpopulation size patterns are not qualitatively influenced by inclusion of sex as a factor in the analyses of the smaller set of populations where sex data were available (data not shown).

Collected fish were overanesthetized with MS 222 (tricaine methanesulfonate) at the site of capture and stored in 96% ethanol for about two months. After this, fish were moved to 4% formalin. Standard length (from the tip of the nose to the end of the tail base) was measured with a digital calliper to the nearest 0.01 mm at this time. Age was estimated in a subsample of populations (Helsinki, Oulu, Tuolpujärvi, Krugloje, Bynästjärnen, Pyöreälampi, and Rytilampi) from fin rays (and verified from otoliths for one population; e.g., Shirvell 1981). Fins were first cut as near to the base of the fin as possible and then air dried (dorsal, pectoral, or pelvic fin). After that fins were stained with a neutral red solution

Table 1. Sampling sites, their abbreviations, coordinates, sample sizes, surface area of water bodies, and diversity of fish community. Coordinates of the Russian sites are approximate ones based on Ziuganov and Zotin (1995). *Gasterosteus aculeatus* and *Coregonus lavaretus* are larger-bodied competitors, while *Salmo trutta* is a predator of *Pungitius pungitius*

Sampling site	Abbreviation	Coordinates	Sample size (aged)	Surface area (ha)	Fish community
Marine (coastal) populations					
Bölesviken, Baltic Sea	BÖL	63°39′N; 20°12′E	70	N/A	Complex
Oulu, Baltic Sea	OUL	64°58′N; 25°29′E	30 (19)	N/A	Complex
Helsinki, Baltic Sea	HEL	60°13′N; 25°11′E	59 (29)	N/A	Complex
Levin Navolok, White Sea	LEV	66°18′N; 33°25′E	55	N/A	Complex
Lakes					
Kevojärvi	KEV	69°45′N; 27°00′E	40	115	Complex
Pulmankijärvi	PUL	69°58′N; 27°58′E	42	1620	Complex
Tuolpujärvi	YUO	69°34′N; 28°02′E	40 (23)	185	Complex
Porontima	POR	66°12′N; 29°16′E	56	115	Complex
Vastre-Skavtrasket	SKA	64°26′N; 19°27′E	30	35	Complex
Ponds					
Bolotnoje	BOL	66°18′N; 33°25′E	43	<5 (isolated)	*G. aculeatus*[1]
Krugloje	KRU	66°18′N; 33°25′E	32 (26)	<5 (isolated)	*G. aculeatus*[1]
Mashinnoje	MAS	66°18′N; 33°25′E	63	<5 (isolated)	*G. aculeatus*[1]
Pyöreälampi	PYÖ	66°15′N; 29°26′E	158 (21)	<5 (isolated)	*C. lavaretus*[2]
Rytilampi	RYT	66°23′N; 29°19′E	115 (25)	<5 (isolated)	–
Kirkasvetinenlampi	KIR	66°26′N; 29°08′E	73	<5 (isolated)	*S. trutta*
Abbortjärn	ABB	64°29′N; 19°26′E	80	<5 (isolated)	–
Bynästjärnen	BYN	64°27′N; 19°26′E	91 (30)	<5 (isolated)	–
Hansmyrtjärn	HAN	64°33′N; 19°10′E	61	<5 (isolated)	*S. trutta*

[1] Heavily outnumbering *P. pungitius*.
[2] In extremely low numbers.

(with acetic acid). Annuli were evaluated under microscope with 30–100× magnification. Note that for the purpose of age determination, individuals were chosen to cover the full size range within populations. Hence, due to nonrandom sampling, the age data cannot be used for direct assessment of age structure. In the case of four populations (Oulu, Tuolpujärvi, Kevojärvi and Pulmankijärvi, see Table 1 for details), fish were stored in 96% ethanol for about three years before the samples were moved to formalin. Even though storage in alcohol can cause some shrinkage, length becomes stable during less than two months (e.g., Fox 1996; Kristoffersen and Salvanes 1998). Further, the length change was found to be minor (<3%) in fish species comparable in size to *P. pungitius* (Kristoffersen and Salvanes 1998). Hence, this together with the fact that all samples were stored in alcohol for at least two months suggests that our samples were comparable.

COMMON GARDEN EXPERIMENT

Four geographically isolated populations, representing two marine (Helsinki, Baltic Sea, and Levin Navolok, White Sea) and two pond (Bynästjärnen and Pyöreälampi, separated by >500 km) habitats were chosen for the common garden experiment (Fig. 1). The marine sites were shallow coastal bays close to creek inlets and thus, they represented low salinity marine habitats (Baltic Sea being a brackish water environment in general). Adult fish were collected before or during the early phase of the breeding season in 2007 and transported to the aquacultural facilities of the University of Helsinki. Fish were provided with frozen bloodworms (Chironomidae sp.) and kept at 17°C and a 24 h light period until enough fish from each population had turned to reproductive condition. Both the collected adults and their offspring were kept and reared in freshwater.

Five full-sib families were made *in vitro* from each of the four populations. The clutches were transported to 1.4 L tanks of two Allentown Zebrafish Rack Systems (Aquaneering Inc., San Diego, CA, USA). Racks were equipped with a multilevel filtering system including UV, physical, chemical, and biological filters, and had closed water circulation. Four days after hatching, when the fry started to swim freely, 10 fish from each family were randomly chosen and housed individually in the 1.4 L tanks, summing to 200 individually kept fish (four populations × five families × 10 individuals). Visual contact between the tanks was blocked. Fish were fed first with live brine shrimp (*Artemia salina*) nauplii, and then with frozen copepods (*Cyclops* sp.) and

frozen bloodworms twice a day ad libitum. Temperature was set to 17°C throughout the experiment. We started with a 24 h light period (representative to high latitudes at summer) and changed it to a 12:12 h light–dark periodism gradually during one week after 12 weeks. Due to latitudinal differences between the source populations (Fig. 1), we did not aim to mimic the natural light regimes any closer and did not attempt to follow natural temperature regimes.

The fish were measured at the age of 36 weeks, when the growth curves of all populations approached their asymptotes (Herczeg et al. unpubl. data). Standard length was measured from digital photographs taken under standardized conditions with a ruler placed in each photograph for scaling using tpsDig 1.37 (Rohlf 2002) software. Body weight was taken to the nearest 0.01 g with a digital balance. Due to mortality, and because some randomly chosen fish were sacrificed earlier for other scientific purposes, we could use 86 fish, 21 from the Baltic Sea (family representations: 6, 5, 4, 3, 3), 23 from the White Sea (family representations: 7, 6, 5, 3, 2), 20 from Bynastjärnen (family representations: 7, 4, 3, 3, 3), and 22 from Pyöreälampi (family representations: 6, 5, 5, 5, 1). We note that although the use of the F1 laboratory generation should remove a large part of environmental effects on growth, some maternal effects or cross-generational influences may remain. However, most maternal effects on growth dissipate quickly and seldom explain any large interpopulational differences in growth (e.g., Green 2008).

STATISTICAL ANALYSIS

We analyzed our data with the aid of General Linear Mixed Models (GLMMs) as implemented in SAS (Littell et al. 2006). In the first GLMM, we analyzed body size differences between habitat types. Standard length was the dependent variable (fresh weight was not available, while the differences in alcohol storage might bias weight measures considerably), habitat type (marine, lake, or pond) a fixed factor, and population nested within the habitat type a random factor. As most of our populations were isolated from each other, we did not enter geographic distances into our model. The analysis of the effect of recently introduced predators were based on a subset of the populations: a lake, a predator free pond, and a pond with introduced *S. trutta*—all being in close geographic proximity (Fig. 1). This setup was replicated in the Kuusamo region in Finland and the Åmsele region in Sweden. The ponds with introduced predators were Kirkasvetinenlampi in the Kuusamo and Hansmyrtjärn in the Åmsele region (Fig 1). Both regions had two ponds without predators (Bynästjärnen and Abbortjärn in the Åmsele region and Pyöreälampi and Rytilampi in the Kuusamo region), but we included only one pond per region for a symmetric setup. However, to provide a conservative comparison, we chose the pond population with the smaller mean body size in both cases (Abbortjärn and Pyöreälampi, see Figs. 1 and 2). Unfortunately, we are not aware of any other similar population system (i.e., one pond + one pond with introduced predator + one lake within a small geographic area), so our results based on only two replicates should be treated with caution. We used a GLMM again; standard length was set as the dependent variable, habitat type (lake, pond, pond with introduced *S. trutta*) as a fixed factor, and region (Kuusamo and Åmsele) as a random factor. Age–size relationships between habitats were compared with the third GLMM. The standard length was set as the dependent variable, habitat type as a fixed factor, population nested within habitat type as a random factor, and age as a covariate. The size and weight of the common garden experiment fish were analyzed with two GLMMs. The standard length or body weight was set as dependent variables, population as a fixed factor, and family nested within population as a random factor.

All analyses were performed with the aid of the SAS 9.1 (SAS Institute, Cary, NC, USA).

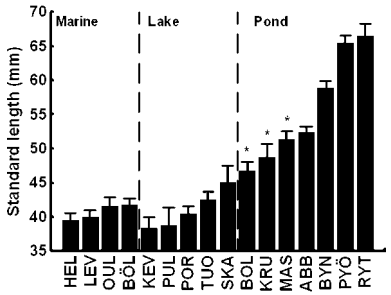


Figure 2. Mean standard length (+95% CI) of wild caught nine-spined sticklebacks in different population and habitat types. For the population abbreviations see Table 1. The star symbols mark the recently isolated *P. pungitius* populations where they are heavily outnumbered by *G. aculeatus*.

Results

BODY SIZE TRENDS

The sticklebacks from different habitat types differed significantly in their standard length (GLMM: $F_{2,13.1} = 13.51$, $P < 0.001$; least-squares means ± S.E., all in mm: marine = 40.72 ± 2.84, lake = 41.02 ± 2.54, pond = 55.74 ± 2.14; Fig. 2). Post hoc (Scheffe) tests revealed that while marine and lake fish did not differ ($P = 0.99$), both were smaller than pond fish (marine–pond: $P = 0.004$; lake–pond: $P = 0.003$). The habitat differences were profound; pond fish were in general 35–40% longer than marine or lake fish. However, the population effect within habitat type was also significant ($\chi^2_1 = 569.8$, $P < 0.001$), mainly due to the large variation among ponds (Fig. 2). While sticklebacks from all pond populations tended to be larger than their marine or lake conspecifics, some pond populations (Bynästjärnen, Pyöreälampi, and Rytilampi) contained adult fish that were strikingly larger than "ordinary" fish from the marine or lake sites.

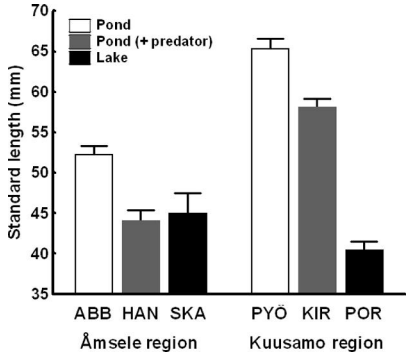


Figure 3. Mean standard length (+95% CI) of nine-spined sticklebacks in different populations, habitat types, and regions. For the population abbreviations, see Table 1.

Standard length of *P. pungitius* differed also between lakes, ponds, and ponds with introduced *S. trutta* (GLMM: $F_{2,454} = 269.13$, $P < 0.001$). Post hoc (Scheffe) tests showed that the habitat types differed in all possible combinations ($P < 0.001$ in all tests; Fig. 3). Ponds with introduced *S. trutta* housed smaller *P. pungitius* than the ponds lacking this predator (Fig. 3; least-squares means ± S.E., all in mm: lake = 40.56 ± 5.10, pond = 59.35 ± 5.07, pond with *S. trutta* = 51.31 ± 5.08). The effect of the region of origin was also significant ($\chi^2_1 = 194.2$, $P < 0.001$): *P. pungitius* in ponds with *S. trutta* differed from lake conspecifics in the Kuusamo region, but not in the Åmsele region (Fig. 3).

AGE–SIZE RELATIONSHIPS

A GLMM revealed a habitat-specific age effect on standard length (habitat: $F_{2,10.8} = 1.28$, $P = 0.32$; age: $F_{1,163} = 46.62$, $P < 0.001$; habitat*age: $F_{2,163} = 10.43$, $P < 0.001$; Fig. 4). The population effect within habitat type was significant ($\chi^2_1 = 48.5$, $P < 0.001$). To test for habitat effects directly, we removed the interaction term. Here, habitat was only significant when we removed Krugloje, a pond where *P. pungitius* is found in sympatry with *G. aculeatus* (with Krugloje, habitat: $F_{2,4.23} = 2.26$, $P = 0.21$; age: $F_{1,167} = 253.94$, $P < 0.001$; without Krugloje, habitat: $F_{2,3.61} = 34.00$, $P < 0.005$; age: $F_{1,141} = 226.32$, $P < 0.001$; Fig. 4). The significant habitat effect (after the exclusion of Krugloje) suggests that body size is different at a given age between habitats when we consider only pond *P. pungitius* populations without competitor or predatory fish species being present, while the significant interaction term (it remained highly significant after removing Krugloje, data not shown) suggests that the effect of age on size is habitat specific (Fig. 4). Old fish from Pyöreälampi and Rytilampi ponds were two times longer than fish from lakes or marine sites, a phenomenon we recognize as gigantism. Interestingly, second year fish were not represented among the breeding adults of the ponds.

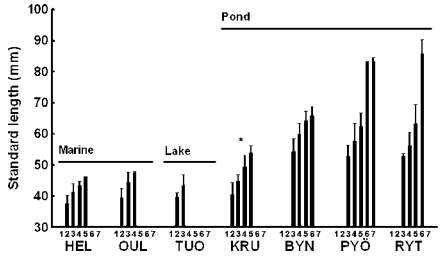


Figure 4. Mean size (+95% CI) of nine-spined sticklebacks at given ages from different populations and habitat types. For the population abbreviations see Table 1. The numbers above the population abbreviations denote age in years. The star symbols mark a recently isolated *P. pungitius* population where nine-spined sticklebacks are heavily outnumbered by *G. aculeatus*.

COMMON GARDEN EXPERIMENT

The analyses of standard length and body weight variation from the common garden experiments revealed concurrent patterns: populations differed significantly both in standard length ($F_{3,14.9} = 60.46$, $P < 0.001$; least-squares means ± S.E., all in mm: Helsinki [Baltic Sea] = 46.52 ± 1.49, Levin Navolok [White Sea] = 60.80 ± 1.48, Bynästjärnen = 68.98 ± 1.51; Pyöreälampi = 72.74 ± 1.51; Fig. 5) and body weight ($F_{3,14.1} = 81.50$, $P < 0.001$; least-squares means ± S.E., all in g: Helsinki [Baltic Sea] = 0.91 ± 0.13, Levin Navolok [White Sea] = 1.69 ± 0.13, Bynästjärnen = 3.32 ± 0.13; Pyöreälampi = 3.09 ± 0.13; Fig. 5). Post hoc tests showed that Levin Navolok fish (White Sea) were longer and heavier than Helsinki fish (Baltic Sea; standard length: $P < 0.001$; body weight: $P = 0.006$). The pond populations (Bynästjärnen and Pyöreälampi) did not differ from each other (standard length: $P = 0.41$; body weight: $P = 0.66$), while fish from both marine populations were smaller than fish from both ponds (all $P < 0.015$). Size differences were profound: pond fish grew two to three times heavier than marine fish during 36 weeks (Fig. 5). The family effect was significant both in the case of standard length ($\chi^2_1 = 8.1$, $P < 0.005$) and body weight ($\chi^2_1 = 4.1$, $P < 0.05$).

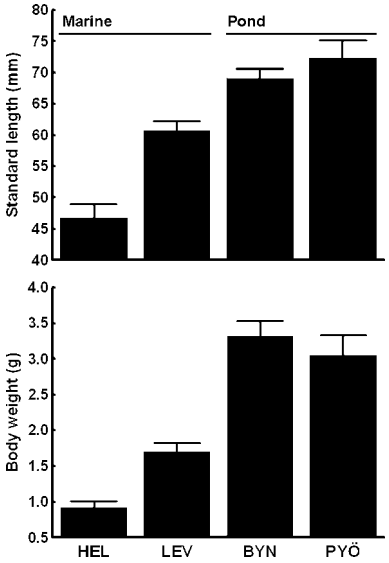


Figure 5. Mean (+95% CI) standard length and body weight of 36 weeks old individually reared common garden fish in different populations and habitats. For the population abbreviations, see Table 1.

Discussion

According to theory, when the selective forces stemming from predation and interspecific competition are relaxed, small organisms should evolve to become larger because several forms of intraspecific competition and sexual selection all favor larger body size. By studying a widely distributed small-bodied fish species, we found support for this theory in an aquatic vertebrate. *P. pungitius* has a standard length around 4 cm (about 5–6 cm in total length) in habitats with diverse fish communities (e.g., Bănărescu and Paepke 2001). We found that it grew larger in isolated ponds, and turned into 8–9 cm (up to 10–12 cm in total length) giants in some populations. *P. pungitius* in ponds with introduced predatory *S. trutta* or sympatric competitor *G. aculeatus* were smaller than those in predatory and competitor fish-free ponds. Our size-at-age analyses demonstrated that the increased size was a result of both extended longevity and increased growth rates in the natural pond environments. Fish in the common garden study showed similar size trends to their conspecifics from the wild, pond fish reaching two to three times larger body mass than their marine conspecifics during 36 weeks of growth. This finding strongly suggests that the body size trends seen in the data from the wild have a genetic basis. The repeated, habitat-specific nature of the genetically based body size shifts suggests that observed divergence is adaptive and driven by natural selection (e.g., Clarke 1975; Endler 1986). Obviously, we cannot derive conclusions for the generality of the island rule in fish as we studied one species only. Our results, however, fit perfectly to the predictions drawn from the island rule: in the island-like ponds, where gene flow was zero, interspecific competition and predation negligible, *P. pungitius* was larger (sometimes reaching giant sizes) than that in the mainland-like large lakes or marine sites.

Apart from sexual selection, which generally favors larger body size within a population (e.g., Wooton 1979; Andersson 1994), several other agents of natural selection—or their absence—can influence the mean body size in a population. For instance, increasing population density and intraspecific competition as a consequence can lead to an increase in body size (Clegg and Owens 2002; Wu et al. 2006, but see, e.g., Boucher et al. 2004 for an opposite pattern in the case of resource limitation). This effect, coupled with that of sexual selection, can be expected to act particularly strongly in cases when predation acting against large or fast-growing phenotypes (Blanckenhorn 2000; Biro et al. 2004, 2006) or interspecific competition narrowing the population's niche (Wilson 1975; Lomolino 1985; Simberloff et al. 2000) are relaxed, a phenomenon typical on islands (McNab 1994; Lomolino 2005). These expectations match our observations: the largest sizes were observed in the Finnish and Swedish ponds where interspecific competition was negligible and predatory pressure low. We did not estimate population densities, but based on our catches, we believe that the densities of *P. pungitius* are much (by magnitudes) higher in ponds than those in the larger habitat types. Evidence for increased intraspecific competition in pond *P. pungitius* populations is also available: we found that the levels of aggression and drive to feed, and the social costs of group living are higher among pond than among marine fish as revealed by common garden experiments (Gonda et al. 2009a; Herczeg et al. 2009a,b). Further, brain parts relevant in chemical communication, habituation, or learning were found to be relatively smaller in pond than in marine sticklebacks (Gonda et al. 2009b). Another factor facilitating local adaptation in general and characteristic of island environments in particular is lack of gene flow (Whittaker 1998). This factor is also relevant in our case: all noncoastal ponds were found to be genetically highly differentiated and isolated from all other populations (Shikano et al. unpubl. data). Hence, considering that all the possible ancestor population types (marine or lake) consist of uniformly small sticklebacks (present study, see also Bănărescu and Paepke 2001; Östlund-Nilsson et al. 2007), repeated evolution of the large, fast growing phenotype seems to have happened.

It has been demonstrated in a series of studies focusing on the predation-related life-history evolution of guppies (*Poecilia reticulata*) that high-predation environments select for earlier maturation at smaller size and larger reproductive allotment (more frequent reproduction, larger number of smaller offspring) than low-predation environments (e.g., Reznick 1982; Reznick and Endler 1982). This pattern has evolved as a response to differences in age-specific mortality: high-predation environments are characterized by high mortality rates that are uniformly distributed across age classes while low-predation environments are characterized by lower mortality rates with relatively higher proportion of mortality prior maturity (Reznick et al. 1996). However, predation-related size divergence was not found in this system (Hendry et al. 2006). It is noteworthy that in the case of three-spined sticklebacks in which the dorsal and pelvic spines together with the supporting bony lateral plates provide effective means of antipredator defense (e.g., Hoogland et al. 1957; Reimchen 1983), large body size can serve fish to escape from gape-limited predators, and as a consequence, the presence of gape-limited predators can select for gigantism (e.g., Moodie 1972a,b; Moodie and Reimchen 1976; Reimchen 1988, 1991). However, the same would not work with *P. pungitius* because their spines are far less effective against predators (Hoogland et al. 1957). Further, in our case, the larger body size of pond *P. pungitius* is found parallel to the reduction or even complete loss of the pelvic apparatus in the absence of predatory fish (Herczeg et al. unpubl. data).

Among the ponds, the Russian coastal populations were the closest to marine and lake populations in body size, and the observed size differences appeared to be a result of extended longevity only. However, one of the factors expected to be responsible for insular body size evolution, namely the relaxation of interspecific competition, did not hold true due to the large numbers of sympatric larger bodied three-spined stickleback in these ponds. The recent isolation of these ponds from the White Sea and, as a consequence, the lack of genetic divergence from their marine conspecifics (Shikano et al. unpubl. data) is less likely to be the cause, because the time since isolation from the White Sea was enough for *P. pungitius* to lose/reduce its pelvic apparatus in these ponds (Ziuganov and Zotin 1995; Herczeg et al. unpubl. data). Hence, while evolution has clearly already occurred in these ponds, it does not apply to body size. This together with the fact that ponds with introduced predatory fish (*S. trutta*) exhibited lower or negligible size divergence from the marine and lake populations suggests that from the point-of-view of body size evolution, the biotic environment (i.e., predation and competition) is more important than the area of the habitat per se.

Reporting phenotypic body size trends from the wild does not tell us much about the functional (i.e., increased growth rate vs. increased longevity), or the evolutionary mechanism (i.e., local adaptation or phenotypic plasticity) behind the observed patterns. Analyzing size-at-age relationships showed that in the case of the Finnish and Swedish ponds, the maximal age of *P. pungitius* was two to three years higher and fish were also considerably larger at the same age than in marine or lake environments. In other words, pond fish grew faster and for longer periods than the marine and lake fish. Again, the Russian pond (Krugloje) was an exception: the size at a given age was similar to the marine and lake fish, while longevity was extended. This extended longevity might result simply from the low predation-caused mortality. However, it is noteworthy that—excluding Krugloje—the sizes at a given age were very consistent within the marine and lake fish and within the Finnish and Swedish ponds despite the often considerable geographic and genetic distances. Interestingly, the real giant fish were found in the sixth and seventh year classes in Pyöreälampi and Rytilampi showing a boost in growth after the fifth year. Intuitively, it might suggest some generation-specific environmental effects, but the fact that the existence of giant fish above 10 cm in total length in these populations has been observed during several years (Kuusela 2006; Merilä 2006; personal observations) contradicts this scenario. The cause and relevance of this "terminal gigantism" surely warrants further investigations. It is also noteworthy that we did not catch a single second year fish in the Finnish and Swedish ponds. The reason for this is unclear, but it might be that fish in the giant populations initiate breeding a year later—another pattern warranting further studies on the population dynamics of the giant populations. At any rate, this difference alone was clearly not enough to explain the habitat differences in body size.

Proving that the size differentiation observed in this study has a genetic basis is very important for any evolutionary inference, especially as it has been shown that divergence in certain ecological factors between island and mainland environments can result in profound intraspecific body size differentiation via phenotypic plasticity alone (e.g., Madsen and Shine 1993). In the common garden experiment, we found that pond fish grew two to three times larger in size than marine fish, irrespective of population origin. The four populations used in the common garden experiment are also known to be genetically isolated (Shikano et al. unpubl. data), and the within-habitat type (cf. pond vs. marine) replicates were separated by >500 km geographically. Such genetically based, repeated, and habitat-specific divergence strongly implies natural selection as the causal agent (e.g., Clarke 1975; Endler 1986; Schluter and Nagel 1995; McGuigan et al. 2005). The significant family effects within populations further suggest that there is also genetic variation within population in body size. Because first laboratory generation full-sib families were used, maternal and nonadditive effects cannot be isolated from additive genetic effects which need to be estimated with other types of breeding design (e.g., Lynch and Walsh 1998). However, considering the strength of differences (up to threefold, measured

in 36-week-old fish), maternal and/or nonadditive effects are unlikely to account for the observed differentiation considering that maternal effects seldom explain any large interpopulation differences in growth and dissipate quickly during the early growth phase (e.g., Green 2008). Further, adult fish were kept at ad libitum food for weeks before the crosses were made, meaning that they did not face any energy limitation during that period.

In summary, we found that a small-sized teleost fish, *P. pungitius*, was generally larger in isolated ponds than in lakes or marine environments, sometimes reaching giant sizes. We demonstrated that this was a result of a combination of extended longevity and faster growth in the wild, and also that these patterns are likely to be genetically based as verified by a common garden experiment. We suggest that the relaxation of predation pressure and interspecific competition together with the increased intraspecific competition—and not habitat area per se—are the key factors behind insular body size evolution. Approaches such as those adopted in this study can provide important and complementary insights into the mechanisms—as well as their validity and generality—underlying body size–and island size correlations. According to Lomolino (2005), very large islands are "mainland like" in terms of predators and competitors. We propose that a single predator or larger and superior interspecific competitor species can transform even the smallest island into a "mainland like" habitat from the perspective of the selective pressures relevant for body size evolution.

ACKNOWLEDGMENTS

We thank Victor Berger, Göran Englund, Tuomas Leinonen, Daniel Lussetti, and Pirkko Siikamäki for their help in obtaining the samples. We are indebted to the Oulanka Research Station (University of Oulu) and the White Sea Biological Station (Russian Academy of Sciences) for their help with practicalities in the field. We thank Anders Forsman, Mark Lomolino, Shai Meiri, Loeske Kruuk, and three anonymous reviewers for their thorough comments leading to improvements of the manuscript, and John Loehr for correcting the English. We received financial support from the Academy of Finland, Ministry of Education and Centre for International Mobility (CIMO). The experiment was conducted under the license of the Helsinki University Animal Experimentation Committee.

LITERATURE CITED

Andersson, M. 1994. Sexual selection. Princeton Univ. Press, Princeton, NJ.

Bănărescu, P. M., and H.-J. Paepke. 2001. The freshwater fishes of Europe. Vol. 5/III. AULA, Wiebelsheim.

Biro, P. A., M. V. Abrahams, J. R. Post, and E. A. Parkinson. 2004. Predators select against high growth rates and risk-taking behaviour in domestic trout populations. Proc. Roy. Soc. Lond. B 271:2233–2237.

———. 2006. Behavioural trade-offs between growth and mortality explain evolution of submaximal growth rates. J. Anim. Ecol. 75:1165–1171.

Blanckenhorn, W. U. 2000. The evolution of body size: what keeps organisms small? Q. Rev. Biol. 75:385–407.

Boback, S. M. 2003. Body size evolution in snakes: evidence from island populations. Copeia 2003:81–94.

Boback, S. M., and C. Guyer. 2003. Empirical evidence for an optimal body size in snakes. Evolution 57:345–351.

Boucher, S., M. Crete, J. P. Ouellet, C. Daigle, and L. Lesage. 2004. Large-scale trophic interactions: White-tailed deer growth and forest understory. Ecoscience 11:286–295.

Case, T. J. 1978. A general explanation of insular body size trends in terrestrial vertebrates. Ecology 59:1–18.

———. 1979. Optimal body size and an animal's diet. Acta Biotheor. 28:54–69.

Clegg, S. M., and I. P. F. Owens. 2002. The 'island rule' in birds: medium body size and its ecological implications. Proc. Roy. Soc. Lond. B 269:1359–1365.

Clarke, B. C. 1975. The contribution of ecological genetics to evolutionary theory: detecting the direct effects of natural selection on particularly polymorphic loci. Genetics 79(Suppl.):101–113.

Clutton-Brock, T. H., F. E. Guiness, and S. D. Albon. 1982. Red deer: behaviour and ecology of two sexes. Univ. of Chicago Press, Chicago, IL.

Damuth, J. 1993 Cope's rule, the island rule and the scaling of mammalian population density. Nature 365:748–750.

Endler, J. A. 1986. Natural Selection in The Wild. Princeton Univ. Press, Princeton, NJ.

Fairbairn, D. J., and R. F. Preziosi. 1994. Sexual selection and the evolution of allometry for sexual size dimorphism in the water strider, *Aquarius remigis*. Am. Nat. 144:101–118.

Forsman, A. 1991. Variation in sexual size dimorphism and maximum body size among adder populations: effects of prey size. J. Anim. Ecol. 60:253–267.

Foster, J. B. 1964. Evolution of mammals on islands. Nature 202:234–235.

Fox, C. J. 1996. Length changes in herring (*Clupea harengus*) larvae: effects of capture and storage in formaldehyde and alcohol. J. Plankton Res. 18:483–493.

Gittleman, J. L. 1985. Carnivore body size: ecological and taxonomic correlates. Oecologia 67:540–554.

Gonda, A., G. Herczeg, and J. Merilä. 2009a. Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain. Proc. Roy. Soc. B. 276:2085–2092.

Gonda, A., G. Herczeg, and J. Merilä. 2009b. Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? J. Evol. Biol. 22:1721–1726.

Grant, P. R. 1998. Evolution on islands. Oxford Univ. Press, New York.

Green, B. S. 2008. Maternal effects in fish populations. Adv. Mar. Biol. 54: 1–105.

Heaney, L. R. 1978. Island area and body size of insular mammals: evidence from the tri-colored squirrel (*Callosciurus prevosti*) of Southeast Asia. Evolution 32:29–44.

Hendry, A. P., E. B. Taylor, and J. D. McPhail. 2002. Adaptive divergence and the balance between selection and gene flow: lake and stream stickleback in the Misty system. Evolution 56:1199–1216.

Hendry, A. P., M. L. Kelly, M. T. Kinnison, and D. N. Reznick. 2006. Parallel evolution of the sexes? Effects of predation and habitat features on the size and shape of wild guppies. J. Evol. Biol. 19:741–754.

Herczeg, G., A. Gonda, and J. Merilä. 2009a. Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). J. Evol. Biol. 22:544–552.

———. 2009b. The social cost of shoaling covaries with predation risk in nine-spined stickleback (*Pungitius* pungitius) populations. Anim. Behav. 77:575–580.

Hoogland, R., D. Morris, and N. Tinbergen. 1957. The spines of sticklebacks (*Gasterosteus* and *Pygosteus*) as means of defence against predators (*Perca* and *Esox*). Behaviour 10:205–236.

Jones, J. W., and H. B. N. Hynes. 1950. The age and growth of *Gasterosteus aculeatus*, *Pygosteus pungitius* and *Spinachia vulgaris* as shown by their otoliths. J. Anim. Ecol. 19:59–73.

Kahilainen, K., T. Malinen, A. Tuomaala, and H. Lehtonen. 2004. Diel and seasonal habitat and food segregation of three sympatric *Coregonus lavaretus* forms in a subarctic lake. J. Fish Biol. 64:418–434.

Keogh, J. S., I. A. W. Scott, and C. Hayes. 2005. Rapid and repeated origin of insular gigantism and dwarfism in Australian tiger snakes. Evolution 59:226–233.

Kingsolver, J. G., and D. W. Pfennig. 2004. Individual-level selection as a cause of Cope's rule of phyletic size increase. Evolution 58:1608–1612.

Kristoffersen, J. B., and A. G. V. Salvanes. 1998. Effects of formaldehyde and ethanol preservation on body and otoliths of *Maurolicus muelleri* and *Benthosema glaciale*. Sarsia 83:95–102.

Kuusela, K. 2006. Isolated ninespine stickleback population, *Pungitius pungitius* (L.) in northeast Finland: large fish with reduced pelvis. Verh. Internat. Verein. Limnol. 29:1409–1412.

Lawlor, T. E. 1982. The evolution of body size in mammals: evidence from insular populations in Mexico. Am. Nat. 119:54–72.

Littell, R. C., G. A. Milliken, W. W. Stroup, R. D. Wolfinger, and O. Schabenberger. 2006. SAS for mixed models, 2nd ed. SAS Institute Inc., Cary, NC.

Lomolino, M. V. 1985. Body size of mammals on islands: the island rule re-examined. Am. Nat. 125:310–316.

———. 2005. Body size evolution in insular vertebrates: generality of the island rule. J. Biogeogr. 32:1683–1699.

Lomolino, M. V., D. F. Sax, B. R. Riddle, and J. H. Brown. 2006. The island rule and a research agenda for studying ecogeographical patterns. J. Biogeogr. 33:1503–1510.

Losos, J. B., and D. Schluter. 2000. Analysis of an evolutionary species–area relationship. Nature 408:847–850.

Losos, J. B., T. R. Jackman, A. Larson, K. de Queiroz, and L. Rodríguez-Schettino. 1998. Contingency and determinism in replicated adaptive radiations of island lizards. Science 279:2115–2118.

Lynch, M., and B. Walsh. 1998. Genetics and analysis of quantitative traits. Sinauer Associates, Sunderland.

MacArthur, R. H., and E. O. Wilson. 1967. The theory of island biogeography. Princeton Univ. Press, Princeton, NJ.

Madsen, T., and R. Shine. 1993. Phenotypic plasticity in body sizes and sexual size dimorphism in European grass snakes. Evolution 47:321–325.

Mayr, E. 1967. The challenge of island faunas. Austr. Nat. Hist. 15:369–374.

McClain, C. R., A. G. Boyer, and G. Rosenberg. 2006. The island rule and the evolution of body size in the deep sea. J. Biogeogr. 33:1578–1584.

McGuigan, K., S. F. Chenoweth, and M. W. Blows. 2005. Phenotypic divergence along lines of genetic variance. Am. Nat. 165:32–43.

McNab, B. K. 1994. Resource use and the survival of land and freshwater vertebrates on oceanic islands. Am. Nat. 144:643–660.

Meiri, S. 2007. Size evolution in island lizards. Global Ecol. Biogeogr. 16:702–708.

Meiri, S., T. Dayan, and D. Simberloff. 2004. Body size of insular carnivores: little support for the island rule. Am. Nat. 163:469–479.

Meiri, S., D. Simberloff, and T. Dayan. 2005a. Insular carnivore biogeography: island area and mammalian optimal body size. Am. Nat. 165:505–514.

Meiri, S., T. Dayan, and D. Simberloff. 2005b. Area, isolation and body size evolution in insular carnivores. Ecol. Lett. 8:1211–1217.

———. 2006. The generality of the island rule re-examined. J. Biogeogr. 33:1571–1577.

Meiri, S., N. Cooper, and A. Purvis. 2008. The island rule: made to be broken? Proc. Roy. Soc. B 275:141–148.

Merilä, J. 2006. Ennätyksellisen iso kymmenpiikki (*Pungitius pungitius*) Kusamon Rytilammesta. Luonnon Tutkija 110:91–93.

Moodie, G. E. E. 1972a. Predation, natural selection and adaptation in an unusual threespine stickleback. Heredity 28:155–167.

———. 1972b. Morphology, life history and ecology of an unusual stickleback (*Gasterosteus aculeatus*) in the Queen Charlotte Islands. Can. J. Zool. 50:721–738.

Moodie, G. E. E., and T. E. Reimchen. 1976. Phenetic variation and habitat differences in *Gasterosteus* populations of the Queen Charlotte Islands. Syst. Zool. 25:49–61.

Moore, J.-S., J. L. Gow, E. T. Taylor, and A. P. Hendry. 2007. Quantifying the constraining influence of gene flow on adaptive divergence in the lake-stream three-spine stickleback system. Evolution 61:2015–2026.

Östlund-Nilsson, S., I. Mayer, and F. A. Huntingford. 2007. Biology of the three-spined stickleback. CRC Press, Boca Raton, FL.

Palkovacs, E. P. 2003. Explaining adaptive shifts in body size on islands: a life history approach. Oikos 103:37–44.

Palmer, M. 2002. Testing the 'island rule' for a tenebrionid beetle. Acta Oecol. 23:103–107.

Peters, R. H. 1983. The ecological implications of body size. Cambridge Univ. Press, Cambridge.

Reimchen, T. E. 1983. Structural relationships between spines and lateral plates in threespine stickleback (*Gasterosteus aculeatus*). Evolution 37:931–946.

———. 1988. Inefficient predators and prey injuries in a population of giant stickleback. Can. J. Zool. 66:2036–2044.

———. 1991. Trout foraging failure and the evolution of body size in stickleback. Copeia 1991:1098–1104.

Reznick, D. 1982. The impact of predation on life history evolution in Trinidadian guppies: genetic basis of observed life history patterns. Evolution 36:1236–1250.

Reznick, D., and J. A. Endler. 1982. The impact of predation on life history evolution in Trinidadian guppies (*Poecilia reticulata*). Evolution 36:160–177.

Reznick, D. N., M. J. Butler IV, F. H. Rodd, and P. Ross. 1996. Life-history evolution in guppies (*Poecilia reticulata*): 6. Differential mortality as a mechanism for natural selection. Evolution 50:1651–1660.

Roff, D. A. 1992. The evolution of life histories. Chapmann & Hall, New York.

Rohlf, F. J. 2002. tpsDig, digitize landmarks and outlines, version 1.37. Department of Ecology and Evolution, State University of New York at Stony Brook.

Schluter, D. 2001. The ecology of adaptive radiation. Oxford Univ. Press, Oxford, U.K.

Schluter, D., and L. M. Nagel. 1995. Parallel speciation by natural selection. Am. Nat. 146:292–301.

Shine, R. 1988. The evolution of large body size in females: a critique of Darwin's fecundity advantage model. Am. Nat. 131:124–131.

———. 1989. Ecological causes for the evolution of sexual size dimorphism: a review of the evidence. Q. Rev. Biol. 64:419–461.

Shirvell, C. S. 1981. Validity of fin-ray ageing for brown trout. J. Fish Biol. 18:377–383.

Simberloff, D., T. Dayan, C. Jones, and G. Ogura. 2000. Character displacement and release in the small Indian mongoose, *Herpestes javanicus*. Ecology 81:2086–2099.

Stanley, S. M. 1973. An explanation for Cope's rule. Evolution 27:1–26.

Stearns, S. C. 1992. The evolution of life histories. Oxford Univ. Press, Oxford, U.K.

Van Valen, L. M. 1973a. Body size and the number of plants and animals. Evolution 27:27–35.

———. 1973b. Pattern and the balance of nature. Evol. Theor. 1:31–49.

Wasserzug, R. J., H. Yang, J. J. Sepkoski, and D. M. Raup. 1979. The evolution of body size on islands: a computer simulation. Am. Nat. 114:287–295.

Welch, J. J. 2009. Testing the Island rule: primates as a case study. Proc. Roy. Soc. B. 276:675–682.

Whittaker, R. J. 1998. Island biogeography. Oxford Univ. Press, Oxford, U.K.

Wilson, D. S. 1975. The adequacy of body size as a niche difference. Am. Nat. 109:769–784.

Wooton, R. J. 1979. Energy cost of egg production and environmental determinants of fecundity in teleost fishes. Symp. Zool. Soc. Lond. 44:133–159.

Wu, Z., Y. Li, and B. R. Murray. 2006. Insular shifts in body size of rice frogs in the Zhoushan Archipelago, China. J. Anim. Ecol. 75:1071–1080.

Ziuganov, V. V., and A. A. Zotin. 1995. Pelvic girdle polymorphism and reproductive barriers in the ninespine stickleback *Pungitius pungitius* (L.) from Northwest Russia. Behaviour 132:1095–1105.

Associate Editor: L. Kruuk

doi: 10.1111/j.1420-9101.2008.01674.x

Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*)

G. HERCZEG, A. GONDA & J. MERILÄ

Department of Biological and Environmental Sciences, Ecological Genetic Research Unit, University of Helsinki, Finland

Keywords:

animal personality;
behavioural syndrome;
behavioural type;
common garden;
geographic variation;
temperament.

Abstract

The proximate and ultimate explanations for behavioural syndromes (correlated behaviours – a population trait) are poorly understood, and the evolution of behavioural types (configuration of behaviours – an individual trait) has been rarely studied. We investigated population divergence in behavioural syndromes and types using individually reared, completely predator- or conspecific-naïve adult nine-spined sticklebacks (*Pungitius pungitius*) from two marine and two predatory fish free, isolated pond populations. We found little evidence for the existence of behavioural syndromes, but population divergence in behavioural types was profound: individuals from ponds were quicker in feeding, bolder and more aggressive than individuals from marine environments. Our data reject the hypothesis that behavioural syndromes exist as a result of genetic correlations between behavioural traits, and support the contention that different behavioural types can be predominant in populations differing in predation pressure, most probably as a result of repeated independent evolution of separate behavioural traits.

Introduction

Even though correlations between different behaviours were described some time ago (Huntingford, 1976), it is only recently that the interest has shifted towards studying behaviours in different contexts, and/or different behaviours together (e.g. Verbeek *et al.*, 1994; Dingemanse *et al.*, 2003; Réale & Festa-Bianchet, 2003; Bell, 2005; Dingemanse *et al.*, 2007). Results of these studies indicate that, besides the often considerable amounts of individual plasticity, certain behaviours are not totally context-dependent or independent from each other. In other words, an individual that is more aggressive in competitive situations than others in the population might also be more aggressive towards its own offspring, or bolder towards its predators (e.g. Gosling, 2001). Correlations across contexts and between behaviours within population are often referred to as behavioural syndromes (Sih *et al.*, 2004a,b), temperament (Réale *et al.*, 2007) or, as this phenomenon is in close resemblance to human personality, animal personality (Gosling, 2001). Several studies have supported the link between behavioural syndromes and fitness (for a recent meta-analysis see: Smith & Blumstein, 2008). There is also solid evidence for personality traits like exploratory or coping behaviour to be heritable (e.g. Drent *et al.*, 2003; van Oers *et al.*, 2004, 2005).

There are two popular hypotheses aiming to explain the evolution of behavioural syndromes known as 'constraint' and 'adaptive' hypotheses, respectively (Bell, 2005). First, as genetically correlated traits tend to evolve together (Price & Langen, 1992; Lynch & Walsh, 1998), behavioural syndromes might be characteristic to a species and present across different populations as a result of the constraints of the underlying genetic architecture. On the contrary, the 'adaptive' hypothesis predicts that interpopulation variation in the presence–absence of behavioural syndromes exists as a result of the different selective environments. Testing these hypotheses is not easy because – besides natural selection – stochastic processes (e.g. mutation, drift, founder effects, gene flow) can also cause differences in the correlation structure between populations, while population differences in genetic architecture – and thus in the presence/absence of genetic correlations – cannot be excluded either. Therefore, the 'constraint' hypothesis can be rejected if the studied populations lack behavioural correlations, while support for the 'adaptive' hypothesis would require detection of predictable environment-dependent patterns to exclude stochastic effects. In cases where population differences are detected in an unpredictable, environment-independent fashion, more detailed genetic studies are needed to distinguish between the hypotheses. Riechert & Hedrick (1993) found that boldness and aggression were correlated in two, markedly different *Agelenopsis aperta* (Araneae) populations. However, their results could be explained by both the 'constraint' or 'adaptive' hypotheses. Bell (2005) was able to reject the 'constraint' hypothesis by comparing wild-caught adult three-spined sticklebacks (*Gasterosteus aculeatus*) from two populations, where the population being under heavy predation showed strong behavioural correlations, whereas the other under low predation pressure did not. Similar divergence was found in group-reared first laboratory generation sticklebacks from the same populations (Bell & Stamps, 2004). Brydges *et al.* (2008) studied wild-caught adult three-spined sticklebacks from eight populations and found behavioural correlations only in one, a result contradicting the 'constraint' hypothesis. The only study which has statistically tested if the presence/absence of behavioural syndromes in a population was associated with ecological conditions is that of Dingemanse *et al.* (2007): they provided support for the 'adaptive' hypothesis by comparing wild-caught juvenile three-spined sticklebacks from six predator free and six predation exposed populations. Despite the limited support, different explanations for the adaptive value of behavioural syndromes have already been proposed (Stamps, 2007; Wolf *et al.*, 2007).

Obviously, there are additional possible mechanisms explaining behavioural syndromes. If we accept the facts that (i) behavioural syndromes can be present in one but not in all populations, and (ii) that the pattern corresponds to differences in the environmental conditions among populations (e.g. Bell, 2005; Dingemanse *et al.*, 2007), phenotypic plasticity alone, or genotype * environment interactions can just as well be responsible for the observed differences as genetic differences resulting from local adaptation. Indeed, in the experiment of Bell & Sih (2007), exposure of predator-naïve wild-caught sub-adult three-spine sticklebacks (previously lacking behavioural correlations) to predation resulted in the emergence of behavioural syndromes. This emergence was a result of both the selection imposed by predators as well as the phenotypically plastic response of the prey (Bell & Sih, 2007).

Irrespective of the presence–absence of behavioural correlations, geographic (interpopulation) variation in behaviour is common (Foster, 1999; Foster & Endler, 1999). Many behavioural traits are affected by predation, for instance Magurran & Seghers (1991, 1994) reported that predator inspection, shoaling and aggression all (co)vary with predation pressure in wild guppies (*Poecilia reticulata*). Single behavioural traits have been found to be heritable in many cases (e.g. Breden *et al.*, 1987; Magurran, 1990; Brown *et al.*, 2007). However, as predation (or the lack of it) is expected to impose complex effects on life history trade-offs (e.g. Blanckenhorn, 2000), it might influence a series of different behaviours at the same time. One way of describing individuals from several behavioural aspects is the adoption of the concept of behavioural type, which is 'a particular configuration of behaviours that an individual expresses' (Bell, 2007).

The aim of the present study was to investigate the presence–absence of behavioural syndromes within, and to compare the behavioural types among populations of nine-spined sticklebacks (*Pungitius pungitius* Linnaeus) differing markedly in predation pressure. The nine-spined stickleback is a perfect model for this purpose, as it occurs in a bewildering range of habitats from seas through large lake or river systems to the smallest creeks and ditches, being able to persist in small, isolated ponds as the only fish species (e.g. Bănărescu & Paepke, 2001; Östlund-Nilsson *et al.*, 2007). We compared four geographically (Fig. 1) and genetically (Shikano, Herczeg & Merilä, unpublished work [Correction added on 23 February 2009, after first online publication: conflation of unpublished work author names corrected]) isolated populations (two marine and two pond, the latter lacking predatory fish) looking for answers to three questions. First, do behavioural syndromes exist when individuals lack prior experience with either predators or conspecifics? Second, if so, does the between population pattern of correlations support the 'adaptive' hypothesis? Third, irrespective of the syndromes, do individuals from different populations represent different behavioural types? In order to remove most of the environmental variation, we measured behaviour of laboratory born first generation individuals reared in the absence of any biological interactions (i.e. contact with predators or conspecifics) until they had reached adult size.

Materials and methods

Sampling, breeding and rearing

Adult *P. pungitius* were collected before or during the early phase of the reproduction period (late May–mid June) in 2007. Four populations were sampled (Fig. 1) with the aid of minnow traps and seine nets. Nine-spined sticklebacks in the two marine populations (Baltic Sea [Finland] and White Sea [Russia]) are sympatric to a large number of predatory and nonpredatory fish, while in the small isolated ponds (Bynästjärnen, Sweden and Pyöreälampi, Finland) it is the only fish species with the exception of a few recently introduced small-bodied whitefish (*Coregonus lavaretus*) in Pyöreälampi. At any rate, considering the potential prey of whitefish (e.g. Kahilainen *et al.*, 2004), it can only be a competitor of *P. pungitius*, and as we never caught a single whitefish

Correspondence: Gábor Herczeg, Department of Biological and Environmental Sciences, Ecological Genetic Research Unit, P.O. Box 65, FI-00014 University of Helsinki, Finland.
Tel.: +358 9 191 57807; fax: +358 9 191 57694;
e-mail: gabor.herczeg@helsinki.fi

© 2009 THE AUTHORS. J. EVOL. BIOL. 22 (2009) 544–552
JOURNAL COMPILATION © 2009 EUROPEAN SOCIETY FOR EVOLUTIONARY BIOLOGY

Fig. 1 Map of Fennoscandia showing the location of the sampling sites. Open circles denote small isolated ponds without predatory fish, while filled circles denote marine populations. BAS, Baltic Sea, Finland; WHS, White Sea, Russia; BYN, Bynästjärnen, Sweden; and PYÖ, Pyöreälampi, Finland.

besides thousands of sticklebacks during our extensive sampling in Pyöreälampi, even that effect should be minimal. The marine sites were shallow coastal bays close to creek inlets, and thus, they represented low salinity sea habitats (Baltic Sea being a brackish water environment in general). The surface area of the ponds was less than 5 ha. Adult fish were transported to the aquaculture facilities of the University of Helsinki and kept under 24 h light photoperiod and fed with frozen bloodworms (Chironomidae sp.) until enough fish from each population had reached reproductive condition.

Between 25 June and 1 July, five full-sib crosses were made artificially (by gently squeezing the eggs out from the ripe females, and pouring the sperm solution obtained by mincing the testicles of over-anaesthetized males on the eggs) from each population. Clutches were transported to 1.4 L tanks of two Allentown Zebrafish Rack Systems (hereafter rack, Aquaneering Inc., San Diego, USA). Racks had a closed water circulation system, with multi-level filtering including physical, chemical, biological and UV filters. We regularly checked the clutches under dissecting microscope, and removed the dead or unfertilized eggs. After the fish started to swim freely (four days after hatching) 10 fish from each family (200 individuals) were housed in the two racks individually (the extra fish were used in other experiments). Visual contact between the tanks was blocked by white plastic panels. Individual rearing was important for two main reasons. Firstly, to provide uniform environment for the individuals within population by removing behavioural variation related to previous experience from predatory encounters or aggressive encounters with conspecifics. Secondly, to provide uniform environment for all populations considering the fact that constraints of group living differ markedly between pond and marine *P. pungitius* (Herczeg *et al.*, 2008), most probably as a result of habitat-dependent differences in aggression (present study). While total isolation might be 'unnatural' for *P. pungitius* and lead to 'abnormal' behaviour, we judged the benefits of measuring behaviour unbiased by previous interactions with predators and conspecifics to be a more important consideration. This in particular from the point of view of genetics: individuals have social ranks in groups, and thus, every individual in a group experience a different social environment, which is bound to confound genetic and environmental influences on behaviours. Likewise, keeping fish without the choice of leaving the group could also be argued to be 'unnatural'. While our setup was efficient in removing most of the possible environmental variation and bias by previous experience, we could not statistically control for possible maternal effects – a problem common to all studies utilizing F1-generation offspring.

Fish were fed in excess first with live brine shrimp (*Artemia* sp.) nauplii, and then with frozen copepods (*Cyclops* sp.) and bloodworms. Twenty-four hour light photoperiod, representative for summer conditions at high latitudes, was used until the fish were 12-weeks-old after which the photoperiod was switched gradually (over 1 week) to a 12 : 12 h light : dark periodism. Because of latitudinal differences between the source populations (Fig. 1), we did not aim to mimic the natural light regimes. Water temperature was set to 17 °C throughout the experiment. Apart from some sparse size measurements (10 measurements of each individual, started after hatching) we did for other purposes, fish remained in isolation for approximately 8 months, after which the behavioural experiments started. Chemical cues of conspecifics, however, could not be eliminated in the closed water circulation in the racks. Use of adult fish was important because the population differences in predation-related selective pressures are most prominent at adult stage in our populations; juveniles are predated by insects, insect larvae and adult conspecifics in both environments. Further, Bell & Stamps (2004) reported ontogenetic changes not only in single behaviours but also in behavioural syndromes. Thus, behaviours measured at one stage of development do not necessary predict those in another. As a result of other scientific purposes and some mortality, we could use 82 fish, 18 from the Baltic Sea (family representations: 6, 3, 3, 2, 4), 22 from the White Sea (family representations: 5, 3, 7, 2, 5), 20 from Bynästjärnen (family representations: 3, 3, 4, 3, 7) and 22 from Pyöreälampi (family representations: 1, 6, 5, 5, 5). We assumed that our samples are

representative of the source populations. All fish were in a nonreproductive state (i.e. their behaviour was not affected by reproduction), meaning that their sex could not be determined by eye. We assumed that the sexes were represented equally. All behavioural tests were conducted between 11.00 and 17.00 h. Fish were tested for the given trait in random order. Experimental fish were not fed the day before the given experiment.

Behavioural tests

Drive to feed

Drive to feed was measured in two ways, first as time to feed in the normal environment and second as time to feed in an altered one. The 1.4 L plastic tanks had two holes at their top, one for feeding (closer to the front) and one for the water inlet (closer to the tank's centre). However, there was enough space to feed the fish with a pipette through both holes. First, on February 22 2008, we provided bloodworms (as on normal days, we provided food in excess) with a pipette through the feeding hole at the top of the tanks to the focal fish (in a similar way to the ordinary feedings) and measured the time until the first biting attempt with a stopwatch in seconds. Second, on February 24 2008, just before the feeding, we placed a shiny steel screw (18 cm long, 1 cm in diameter) through the feeding hole in a way that the screw was reaching the bottom of the tank. Considering the dimensions (ca. 15 × 5 × 25 cm, height, width, length, respectively, the shape was not completely regular) of the tanks, the sudden appearance of the screw was envisioned to represent an alteration of the fish's environment. We provided the bloodworms through the hole where the water inlet entered the tank, and measured the time until the first biting. The whole process (i.e. placing the screw and giving the food) took a few seconds. If a fish did not feed during the first 5 min, the experiment was terminated, and the fish was assigned to a time of 300 s as feeding time.

Alteration of the environment significantly increased the feeding time of the individual fish in all populations (Wilcoxon Matched Pairs Tests: $Z > 2.52$, $P < 0.012$) except in Pyöreälampi (Wilcoxon Matched Pairs Test: $Z = 0.92$, $P = 0.357$), so we assumed that the alteration was successful. Originally, we planned to use the feeding time in an altered environment as a measure of boldness, but it did not correlate with the other measures of boldness (see below) in any of the populations (Spearman rank correlations: $-0.07 < r_{s(18–22)} < 0.26$, $P > 0.299$). However, there was a trend for correlation between feeding time in the normal and altered environments in all populations (Spearman rank correlations; Baltic Sea: $r_{s(18)} = 0.58$, $P = 0.011$; White Sea: $r_{s(22)} = 0.69$, $P < 0.001$; Bynästjärnen: $r_{s(20)} = 0.41$, $P = 0.075$; Pyöreälampi: $r_{s(22)} = 0.36$, $P = 0.099$), so finally we treated both measurements as describing 'drive to feed'.

Aggression

Tanks were organised in five rows (20 tanks per row) on both racks. The measured 82 fish were placed in a way that only every second tank held a fish so as that every fish had empty tanks on both sides. As we kept the extra fish produced from each population, we could present stimulus fish from the same age and population to the focal fish. Stimulus fish were placed into a tank at a random side of the focal fish's tank and allowed to settle for 15 min. After this, the plastic panels blocking visual contact at both sides of the focal fish were removed. This was important for separating aggression from the reaction to a novel object (novel empty tank vs. novel tank with a conspecific; we did not observe any interest or attacks towards the novel empty tank). We waited until the first orientation (being head-first towards the stimulus fish with eyes fixed on it) of the focal fish towards the stimulus fish, and then measured the time the focal fish spent with orientation and the number of attacks (sudden bursts often coupled with biting attempts) it made during the next 5 min. Fish that did not orient until 5 min received zero scores for both measures. Aggression measurements were conducted between 26 February and 3 March 2008.

Boldness

For the boldness measurements, focal fish was gently netted out from their holding tanks and placed tail-first into a grey PVC pipe (28 cm long, 3 cm in diameter) filled up with water (one end of the pipe was permanently sealed). The pipe was immediately submerged into an opaque plastic tank (38 × 36 × 62 cm, height, width, length, respectively), filled up with water to the level of 10 cm. There was a 26 × 13 cm white plastic panel (1 mm thick) glued permanently at the bottom of the tank, positioned so that it covered the area right in front of the unsealed end of the PVC pipe. The unsealed end of the pipe was then blocked with a 6 × 6 × 3 cm grey plastic block. Netting the fish, putting it into the pipe, placing the pipe into the plastic tank, and blocking the pipe's unsealed end with the plastic block took ca. 10 s. We let the fish settle for 3 min, after which the plastic block was lifted with the aid of a string. We measured the time until (i) the fish's head and then its (ii) full body left the pipe. If the fish's head did not appear within 10 min we gave a score of 600 s for both measurements. Water in the plastic tank and the PVC pipe was taken directly from the racks' reservoirs. Boldness measurements were done between 9 and 16 March 2008.

Statistical analyses

Within populations tests

To collapse the two variables for every behavioural trait we aimed to estimate (see above) to a single variable

(drive to feed, aggression and boldness), we run three Principal Component Analyses (PCAs) across populations. In all cases, only the first principal component (PC) had an eigenvector > 1, showing a strong positive relationship with the original variables, and describing the main proportion of variation (see Results). The individual PC scores were used in the subsequent analyses. To see if the PC scores obtained this way were useful for the within population tests (see below), we also run PCAs in a similar fashion, but separately for the different populations. The correlation structure was very similar to that found in the across population PCAs: in all cases the first PC strongly and positively correlated with the two original variables, describing the main proportion of the variation (data not shown). Thus, use of the PC scores calculated across the populations was adequate for our purposes.

We used Spearman rank correlation coefficients to test for the existence of behavioural syndromes within populations with sequential Bonferroni correction (Rice, 1989). Statistical comparisons of the correlation coefficients of the same pairs of behaviours across populations were done with χ^2-tests according to Zar (1999, p. 390). Lack of significance in statistical tests could be argued to be due to low statistical power to detect them. This seems unlikely in our case because *a priori* power calculations estimated with a hypothetic N = 20 (our population sample sizes varied between 18 and 22), suggest that our chance to detect biologically significant correlations was reasonable (power [β] = 0.6 for r_s = 0.5; β = 0.8 for r_s = 0.6; β = 0.9 for r_s = 0.7). We did not conduct *a posteriori* power calculations as they are a 1 : 1 function of the *P*-values, and thus, do not provide any extra information about the reliability of nonsignificant tests (e.g. Hoenig & Heisey, 2001). In cases where individuals did not respond to a given treatment (i.e. did not feed, attack the conspecific, leave the refuge, etc.) the maximal time (time to feed, time to leave refuge) or zero score (time spent with orientation, number of attacks) was assigned. In some behavioural variables (Table 1), the ratio of nonresponders exceeded 50% of the tested individuals from a given population, making correlations somewhat problematic. However, as we believe that not responding is actually a type of response (strengthened by the strong habitat specific pattern of the ratio of responders/nonresponders, see Results) we left these individuals in the analyses. Leaving individuals that did not react to the treatment or scored zero in both measures contributing to the estimate of a given behaviour (drive to feed, aggression or boldness; all based on two original variables) out from the correlations did not change the overall picture (the significant negative drive to feed – aggression correlation in the Baltic Sea became nonsignificant, while the marginally significant negative drive to feed – aggression correlation in the Bynästjärnen population became significant [see Results for the correlations]; data not shown).

Among population tests

To compare behavioural types between populations, we first ran a PCA based on all six behavioural variables on data pooled across populations. This was important, as simply comparing the PC scores of the previous separate PCAs (see 'Within population tests' above) would not compare the individual configuration of different behaviours (= behavioural type, Bell, 2007) but the population means of the different behaviours separately. The first two PCs had eigenvectors > 1 and accounted for over 70% of the total variance together (see Results). To compare PC scores between populations, we constructed two separate General Linear Mixed Models (GLMMs) with the PC scores as dependent variables, population as a fixed factor, and family nested within population as a random factor. For pairwise population comparisons, we used Scheffe *post hoc* tests. The problem imposed by individuals not responding to the given treatments during the observation period being assigned to maximal time (see above) made these analyses only more conservative considering that in reality the response time should have been even larger than the assigned value. To test if the ratio of responders/nonresponders was population specific, we applied log-linear models separately for every original behavioural variable coupled with *a posteriori* pairwise χ^2-tests. All statistical analyses were done with the STATISTICA 6.1 for Windows (StatSoft Inc., Tulsa, USA) and SAS 9.1 for Windows (SAS Institute Inc., Cary, USA) software packages.

Table 1 Population differences in the ratio of responders/nonresponders (in parentheses) observed in the original behavioural measurements. Population pairs that did not differ significantly in the χ^2-tests following the loglinear analyses are also shown (all other pairs differed).

	BAS	WHS	BYN	PYÖ	Population pairs not differing
Sample size	18	22	20	22	–
Time to feed in normal environment	8/10	9/13	19/1	21/1	BAS-WHS**; BYN-PYÖ**
Time to feed in altered environment	4/14	4/18	15/5	19/3	BAS-WHS**; BYN-PYÖ**
Time of orientation	11/7	19/3	20/0	22/0	BAS-WHS*; WHS-BYN*; WHS-PYÖ*; BYN-PYÖ*
Number of attacks	10/8	9/13	17/3	21/1	BAS-WHS**; BYN-PYÖ**
Time to head out	14/4	9/13	20/0	22/0	BYN-PYÖ***
Time to body out	14/4	9/13	20/0	22/0	BYN-PYÖ***

BAS, Baltic Sea; WHS, White Sea; BYN, Bynastjärnen; PYÖ, Pyöreälampi.
Note that in the case of 'time to head out' and 'time to body out' the responders and nonresponders were exactly the same individuals in all populations (i.e. they either left the refuge or not).
*0.1 > *P* > 0.05; **P > 0.249; ***formal test impossible due to repeated zero counts in the same categories, populations treated as not differing

Results

Behavioural syndromes

In all three PCAs of the behaviours across populations, the first PC described the given behaviour (drive to feed, aggression and boldness; Table 2). In all cases, the loadings of the original variables were high and positive, and the PCs explained a high proportion of the variance of the original variables (Table 2).

Correlations between the three PCs were tested separately in every population applying the sequential Bonferroni correction. In the Baltic Sea population we found a negative correlation between drive to feed and aggression ($r_{s(18)} = -0.62$, $P = 0.006$), meaning that fish that fed quicker were also more aggressive. Drive to feed and boldness ($r_{s(18)} = 0.21$, $P = 0.408$) or aggression and boldness were not correlated ($r_{s(18)} = -0.11$, $P = 0.651$). In the other populations none of the correlations were significant (White Sea: drive to feed–aggression, $r_{s(22)} = -0.34$, $P = 0.125$; drive to feed–boldness, $r_{s(22)} = 0.06$, $P = 0.804$; aggression and boldness, $r_{s(22)} = -0.19$, $P = 0.401$; Bynästjärnen: drive to feed–aggression, $r_{s(20)} = -0.43$, $P = 0.056$; drive to feed–boldness, $r_{s(20)} = 0.04$, $P = 0.879$; aggression and boldness, $r_{s(20)} = -0.08$, $P = 0.734$; Pyöreälampi: drive to feed–aggression, $r_{s(22)} = -0.13$, $P = 0.563$; drive to feed–boldness, $r_{s(22)} = 0.09$, $P = 0.706$; aggression and boldness, $r_{s(22)} = -0.18$, $P = 0.414$). Correlation coefficients did not differ among the different populations (drive to feed–aggression, $\chi^2_3 = 3.05$, $P > 0.250$; drive to feed–boldness, $\chi^2_3 = 0.29$, $P > 0.950$; aggression and boldness, $\chi^2_3 = 0.15$, $P > 0.975$) indicating a lack of correlation structure across populations.

Table 2 Results of three principal component analyses of the different behavioural traits conducted by pooling the data across all populations. Loadings, eigenvalues and the percentage of explained variance is provided for each principal component (PC). Only PCs with eigenvalues > 1 are shown. See text for the description of the original variables.

	Original variables	PC1
Drive to feed	Time to feed in normal environment	0.91
	Time to feed in altered environment	0.91
	Eigenvalue	1.67
	% variance	83.30
Aggression	Time of orientation	0.88
	Number of attacks	0.88
	Eigenvalue	1.54
	% variance	76.84
Boldness	Time to head out	0.99
	Time to body out	0.99
	Eigenvalue	1.96
	% variance	98.20

Behavioural types

The PCA run on all behavioural variables across populations resulted in two PCs with eigenvalues > 1 (Table 3). The first PC explained more than half of the variation in behaviours, and gave a biologically interpretable complex description of behaviour: it represented a gradient from quickly feeding, aggressive and bold fish towards slowly feeding, peaceful and shy fish (Table 3). The second PC explained 22% of the variation, and described a somewhat unexpected component of complex behaviour: a gradient from bold and peaceful towards shy and aggressive fish (Table 3).

The GLMM run on the scores of the first PC revealed that families did not differ within populations (Z = 0.53, $P = 0.299$), but populations corrected for the family effects differed significantly in behavioural types ($F_{3,12.5} = 24.47$, $P < 0.001$, Fig. 2a). Furthermore, *post hoc* tests revealed a habitat specific systematic pattern: neither marine ($P = 0.510$), nor small pond populations ($P = 0.896$) differed from each other, while both marine populations differed from both small pond populations (Baltic Sea – Bynästjärnen: $P = 0.005$; Baltic Sea – Pyöreälampi: $P = 0.001$; White Sea – Bynästjärnen: $P < 0.001$; White Sea – Pyöreälampi: $P < 0.001$). In all cases, small pond fish were quicker to feed, more aggressive and bolder than marine fish (Fig. 2a). The GLMM on the second PC revealed that neither families within population (Z = 1.54, $P = 0.062$), nor populations corrected for the family effects differed significantly from each other ($F_{3,15} = 1.60$, $P = 0.231$, Fig. 2b).

The ratio of responders/nonresponders differed significantly between populations in all original behavioural

Table 3 Results of the principal component analysis using all six behavioural variables, based on data pooled across populations. Loadings, eigenvalues and the percentage of explained variance for each principal component (PC) are given. Only PCs with eigenvalues > 1 are shown. See text for the description of the original variables.

Original variables	PC1	PC2
Time to feed in normal environment	–0.75	0.22
Time to feed in altered environment	–0.77	0.12
Time of orientation	0.45	–0.65
Number of attacks	0.71	–0.47
Time to head out	–0.79	–0.55
Time to body out	–0.77	–0.58
Eigenvalue	3.08	1.34
% variance	51.42	22.37

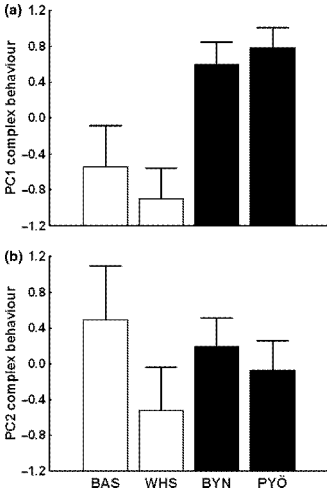


Fig. 2 Population differences in complex behaviours. (a) The first principal component (PC1; 51% of variance explained) describes a gradient from quickly feeding, aggressive and bold individuals (high values) towards reluctant to feed, peaceful and shy individuals (low values). (b) The second principal component (PC2; 22% of variance explained) describes a gradient from bold and peaceful (high values) towards shy and aggressive (low values) individuals. Open bars denote marine, filled bars denote small pond populations. Means + 95% confidence intervals are provided. BAS, Baltic Sea, Finland; WHS, White Sea, Russia; BYN, Bynästjärnen, Sweden; and PYÖ, Pyöreälampi, Finland.

variables as revealed by log-linear analyses (time to feed in normal environment, time to feed in altered environment, time of orientation, number of attacks, time to head out from refuge, time to full body out from refuge; all $\chi^2 > 15.34$, all $P < 0.0015$). Pairwise population comparisons separately for the original variables revealed a strong habitat specific pattern; populations tended to be similar within but different between habitat types (Table 1). In general, marine populations had lower ratio of responders/nonresponders than pond populations.

Discussion

The aim of our experiment was to provide measures of complex behaviour in adult fish unbiased by former experience with predation or aggression from populations that differed markedly in predation pressure. Among the 12 possible behavioural correlations within populations (four populations × three behaviours) we found only one showing a significant trend. Further, we could not detect statistical differences among the correlation coefficients. Hence, the 'constraint' hypothesis of behavioural syndromes was rejected. On the other hand, comparing behavioural types between the different habitats, we found that isolated small ponds were characterized by fish with a higher drive to feed, more aggression and boldness than fish from marine habitats. The habitat dependent occurrence of the same phenotype irrespective of population origin implies natural selection as the causal agent (e.g. Clarke, 1975; Endler, 1986).

The 'constraint' hypothesis evoked to explain the existence of behavioural syndromes predicts strong genetic correlations between different behaviours. This hypothesis can be rejected based on the lack of behavioural correlations. The 'adaptive' hypothesis predicts that correlations between different behaviours might be present in environments where the particular combinations of behaviours are selected for but can be reversed or uncoupled in other environments. The only study testing the predictions of these hypotheses explicitly by statistically comparing the behavioural correlations between high and low predation risk three-spined stickleback populations (six population replicates per habitat type) supported the 'adaptive' hypothesis (Dingemanse *et al.*, 2007). We found minimal evidence for behavioural syndromes. This result clearly contradicts the 'constraint' hypothesis, while the lack of syndromes did not allow us to test the 'adaptive' hypothesis. Our result also contradicts earlier studies reporting numerous behavioural correlations (e.g. Bell & Stamps, 2004; Bell, 2005; Dingemanse *et al.*, 2007). Two possible reasons for the lack of behavioural syndromes are conceivable. Firstly, previous experience with predators or conspecifics is needed for the ontogenetic emergence of behavioural correlations. Bell & Sih (2007) has shown that exposure to predation indeed resulted in the emergence of previously lacking behavioural syndromes, a phenomenon partly caused by plasticity. It is also easy to imagine that dominant individuals within a social group might develop to aggressive, bold and active, whereas subordinates peaceful, shy and inactive. Secondly, the lack of behavioural syndromes in our markedly different populations might simply mean that natural selection did not favour correlated behaviours in any of them. Further experimental studies separating the genetic and environmental contribution to behavioural syndromes following Dingemanse *et al.* (2008), considering both group living and predation are needed.

Geographic variation in behaviour is common (e.g. Foster & Endler, 1999), and there are several studies showing covariation between predation pressure and certain behaviours among populations (e.g. Magurran & Seghers, 1991, 1994; Brown *et al.*, 2005). Predation related behavioural traits have also been shown to be heritable (e.g. Seghers, 1974; Giles, 1984; Magurran, 1990; Brown *et al.*, 2007). However, it also known that

experience has a large (in some cases population specific) effect on the actually expressed behaviours (e.g. Dill, 1974; Magurran, 1990). Our results based on completely conspecific- and predator-naïve *P. pungitius* demonstrate that the studied populations differed in their average behavioural type. We found that fish from the different habitat types differed consistently in a measure of complex behaviour: sticklebacks from ponds without predation were more aggressive, bolder and quicker to feed than their conspecifics from marine habitats. Further, in a somewhat coarse scale, we found that many more pond fish responded to our treatments (i.e. did actually feed, exhibit aggression, or leave the refuge during the observation period) as compared with marine fish. Even though only two replicate populations per habitat type were tested, the large geographical (> 500 km) and genetic (Shikano, Herczeg & Merilä, unpublished work) distance between replicates made them truly independent. It is noteworthy that marine vs. pond habitats differ in several other aspects than predation, for instance in salinity, habitat complexity, interspecific competition, etc., so we can only suggest predation as the most likely factor behind the observed patterns. However, the considerably smaller antipredator defence apparatus (pelvic spines) we observed both in the wild-caught and common garden pond fish when compared with marine fish suggests that the difference in predatory pressure is an important driver of evolutionary divergence between the habitat types. At any rate, the fact that the differences were driven by habitat type and not population origin suggests that the pattern is a result of natural selection (e.g. Clarke, 1975; Endler, 1986; Schluter & Nagel, 1995).

Interestingly, the different behaviours were independent from each other within the populations (= lack of behavioural syndromes), but shifted together in response to presumed differences in the selection pressures across populations. Hence, we suggest that in our case drive to feed, aggression and boldness evolved together, but on an independent genetic basis as a response to the changes in predation pressure. The population differences in complex behaviour might be related to the different life-history strategies of *P. pungitius* populations living in different habitats. Results from our related studies have revealed that pond fish have evolved to grow faster and longer than lake or marine fish, sometimes reaching giant sizes (Herczeg, Gonda & Merilä, unpublished work), and that group living has considerable developmental costs for pond fish, but not for marine fish (Herczeg *et al.*, 2008; Gonda, Herczeg & Merilä, unpublished work). Hence, the life-history evolution of these markedly different and isolated *P. pungitius* populations warrant further investigations.

There are at least two different ways how animals can change their behaviour in response to an increase (or reduction) in predation risk. One would intuitively predict that as the mortality risk imposed by predation increases, prey become shyer and less active, as found by e.g. Bell (2005) and Brydges *et al.* (2008). Interestingly, Brown *et al.* (2005, 2007) reported the opposite: *Brachyraphis episcopii* individuals showed higher activity and were bolder under heavy than low predation risk. The interpretation was that fish in the high predation risk populations have to be bolder to carry on with the necessary activities (Brown *et al.*, 2005, 2007). According to our results, *P. pungitius* behave in the intuitively expected way: increased predation risk makes them risk-averse, and hence, they become shyer and less aggressive, decreasing their feeding activity.

In summary, we found negligible evidence of behavioural syndromes (population level correlations between different behaviours) in first laboratory generation and predator- and conspecific-naïve adult *P. pungitius* from four geographically and genetically isolated populations differing markedly in predation regime. However, the behavioural types (an individual-based estimate of complex behaviour) differed between habitat types, but not between populations within habitat types. Fish from low predation risk populations were quicker feeders, and also more aggressive and bolder than their conspecifics from high predation environments. We suggest that (i) further studies are needed to separate the environmental and genetic components of behavioural syndromes and (ii) that independent evolution of different behavioural traits as a response to different levels of predation can result in population level differences in complex behaviours.

Acknowledgments

Victor Berger, Göran Englund, Tuomas Leinonen, Daniel Lussetti and Pirkko Siikamäki helped us in organizing and executing the field sampling. Special thanks to the Oulanka Research Station and the White Sea Biological Station helping us with the fieldwork. We are highly indebted to Niels Dingemanse for his constructive comments and John Loehr for correcting the English. The authors received financial support from the Academy of Finland and Centre for International Mobility (CIMO). The experiments were conducted under the license of the Helsinki University Animal Experimentation Committee.

References

Bănărescu, P.M. & Paepke, H.-J. 2001. *The Freshwater Fishes of Europe*. Vol. 5/III. AULA-Verlag, Wiebelsheim.

Bell, A.M. 2005. Behavioural differences between individuals and two populations of stickleback (*Gasterosteus aculeatus*). *J. Evol. Biol.* **18**: 464–473.

Bell, A.M. 2007. Future directions in behavioural syndrome research. *Proc. R. Soc. B* **274**: 755–761.

Bell, A.M. & Sih, A. 2007. Exposure to predation generates personality in threespined sticklebacks (*Gasterosteus aculeatus*). *Ecol. Lett.* **10**: 828–834.

Bell, A.M. & Stamps, J.A. 2004. Development of behavioural differences between individuals and populations of sticklebacks ,*Gasterosteus aculeatus*. *Anim. Behav.* **68**: 1339–1348.
Blanckenhorn, W.U. 2000. The evolution of body size: what keeps organisms small? *Q. Rev. Biol.* **75**: 385–407.
Breden, F., Scott, M.A. & Michel, E. 1987. Genetic differentiation for anti-predator behaviour in the Trinidad guppy, *Poecilia reticulata*. *Anim. Behav.* **35**: 618–620.
Brown, C., Jones, F. & Braithwaite, V.A. 2005. In situ examination of boldness-shyness traits in the tropical poeciliid, *Brachyraphis episcope*. *Anim. Behav.* **70**: 1003–1009.
Brown, C., Burgess, F. & Braithwaite, V.A. 2007. Heritable and experiential effects on boldness in a tropical poeciliid. *Behav. Ecol. Sociobiol.* **62**: 237–243.
Brydges, N.M., Colegrave, N., Heathcote, R.J.P. & Braithwaite, V.A. 2008. Habitat stability and predation pressure affect temperament behaviours in populations of three-spined sticklebacks. *J. Anim. Ecol.* **77**: 229–235.
Clarke, B.C. 1975. The contribution of ecological genetics to evolutionary theory: detecting the direct effects of natural selection on particularly polymorphic loci. *Genetics* **79**(Suppl.): 101–113.
Dill, L.M. 1974. The escape response of the zebra danio (*Branchydanio rerio*): the effect of experience. *Anim. Behav.* **22**: 723–730.
Dingemanse, N.J., Both, C., Van Noordwijk, A.J., Rutten, A.L. & Drent, P.J. 2003. Natal dispersal and personalities in great tits (*Parus major*). *Proc. R. Soc. Lond. B* **270**: 741–747.
Dingemanse, N.J., van der Plan, F., Wright, S., Réale, D., Schrama, M., Roff, D.L., van der zee, E. & Barber, I. 2008. Individual experience and evolutionary history of predation affect expression of heritable variation in fish personality and morphology. *Proc. R. Soc. B*, in press.
Dingemanse, N.J., Wright, J., Kazem, A.J.N., Thomas, D.K., Hickling, R. & Dawnay, N. 2007. Behavioural syndromes differ predictably between 12 populations of three-spined stickleback. *J. Anim. Ecol.* **76**: 1128–1138.
Drent, P.J., van Oers, K. & van Noordwijk, A.J. 2003. Realized heritability of personalities in the great tit (*Parus major*). *Proc. R. Soc. Lond. B* **270**: 45–51.
Endler, J.A. 1986. *Natural Selection in the Wild*. Princeton University Press, Princeton.
Foster, S.A. 1999. The geography of behaviour: an evolutionary perspective. *Trends Ecol. Evol.* **14**: 190–195.
Foster, S.A. & Endler, J.A. 1999. *Geographic Variation in Behavior: Perspectives on Evolutionary Mechanisms*. Oxford University Press, New York.
Giles, N. 1984. Development of the overhead fright response in wild and predator naive three-spined sticklebacks, *Gasterosteus aculeatus L*. *Anim. Behav.* **32**: 276–279.
Gosling, S.D. 2001. From mice to men: what can we learn about personality from animal research. *Psychol. Bull.* **127**: 45–86.
Herczeg, G., Gonda, A. & Merilä, J. 2008. The social cost of shoaling covaries with predation risk in ninespine stickleback (*Pungitius pungitius*) populations. *Anim. Behav.* doi: 10.1016/i.anbehar.2008.10.023, in press.
Hoenig, J.M. & Heisey, D.M. 2001. The abuse of power: the pervasive fallacy of power calculations for data analyses. *Am. Stat.* **55**: 19–24.
Huntingford, F.A. 1976. The relationship between anti-predator behaviour and aggression among conspecifics of the three-spine stickleback. *Anim. Behav.* **24**: 245–260.
Kahilainen, K., Malinen, T., Tuomaala, A. & Lehtonen, H. 2004. Diel and seasonal habitat and food segregation of three sympatric *Coregonus lavaretus* forms in a subarctic lake. *J. Fish Biol.* **64**: 418–434.
Lynch, M. & Walsh, B. 1998. *Genetics and Analysis of Quantitative Traits*. Sinauer Associates, Sunderland.
Magurran, A.E. 1990. The inheritance and development of minnow anti-predator behaviour. *Anim. Behav.* **39**: 834–842.
Magurran, A.E. & Seghers, B.H. 1991. Variation in schooling and aggression amongst guppy (*Poecilia reticulata*) populations in Trinidad. *Behaviour* **118**: 214–234.
Magurran, A.E. & Seghers, B.H. 1994. Predator inspection behaviour covaries with schooling tendency amongst wild guppy, *Poecilia reticulata*. *Behaviour* **128**: 121–134.
van Oers, K., Drent, P.J., de Jong, G. & van Noordwijk, A.J. 2004. Additive and nonadditive genetic variation in avian personality traits. *Heredity* **93**: 496–503.
van Oers, K., de Jong, G., van Noordwijk, A.J., Kempenaers, B. & Drent, P.J. 2005. Contribution of genetics to the study of animal personalities: a review of case studies. *Behaviour* **142**: 1191–1212.
Östlund-Nilsson, S., Mayer, I. & Huntingford, F.A. 2007. *Biology of the Three-Spined Stickleback*. CRC Press, Boca Raton.
Price, T. & Langen, T. 1992. Evolution of correlated characters. *Trends Ecol. Evol.* **7**: 307–310.
Réale, D. & Festa-Bianchet, M. 2003. Predator-induced natural selection on temperament in bighorn ewes. *Anim. Behav.* **65**: 463–470.
Réale, D., Reader, S.M., Sol, D., McDougall, P.T. & Dingemanse, N.J. 2007. Integrating animal temperament within ecology and evolution. *Biol. Rev.* **82**: 291–318.
Rice, W.R. 1989. Analyzing tables of statistical tests. *Evolution* **43**: 223–225.
Riechert, S.E. & Hedrick, A.V. 1993. A test for correlations among fitness-linked behavioural traits in the spider *Agelenopsis aperta* (Araneae, Agelenidae). *Anim. Behav.* **46**: 669–675.
Schluter, D. & Nagel, L.M. 1995. Parallel speciation by natural selection. *Am. Nat.* **146**: 292–301.
Seghers, B.H. 1974. Schooling behaviour in the guppy (*Poecilia reticulata*): an evolutionary response to predation. *Evolution* **28**: 486–489.
Sih, A., Bell, A. & Johnson, J.C. 2004a. Behavioural syndromes: an ecological and evolutionary overview. *Trends Ecol. Evol.* **19**: 372–378.
Sih, A., Bell, A.M., Johnson, J.C. & Ziemba, R.E. 2004b. Behavioral syndromes: an integrative overview. *Q. Rev. Biol.* **79**: 241–277.
Smith, B.R. & Blumstein, D.T. 2008. Fitness consequences of personality: a meta-analysis. *Behav. Ecol.* **19**: 448–455.
Stamps, J.A. 2007. Growth-mortality trade-offs and 'personality traits' in animals. *Ecol. Lett.* **10**: 355–363.
Verbeek, M.E.M., Drent, P.J. & Wiepkema, P.R. 1994. Consistent individual differences in early exploratory behaviour of male great tits. *Anim. Behav.* **48**: 1113–1121.
Wolf, M., van Doorn, G.S., Leimar, O. & Weissing, F.J. 2007. Life-history trade-offs favour the evolution of animal personalities. *Nature* **447**: 581–585.
Zar, J.H. 1999. *Biostatistical Analysis*, 4th edn. Prentice-Hall, Upper Saddle River.

Received 16 September 2008; accepted 09 November 2008

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/yanbe

The social cost of shoaling covaries with predation risk in nine-spined stickleback, *Pungitius pungitius*, populations

Gábor Herczeg*, Abigél Gonda, Juha Merilä

Department of Biological and Environmental Sciences, University of Helsinki

ARTICLE INFO

Article history:
Received 8 August 2008
Initial acceptance 19 September 2008
Final acceptance 20 October 2008
Published online 8 January 2009
MS. number: 08-00517R

Keywords:
cost
evolution
grouping
growth
nine-spined stickleback
predation
Pungitius pungitius
schooling
shoaling

The main benefit of grouping is reduced predation risk, while the main costs are thought to derive from competition and increased frequency of social interactions. While the benefits of grouping are well known, its costs have rarely been studied. We studied growth of nine-spined sticklebacks from two marine (high-predation) and two pond (low-predation) populations by rearing them either individually or in groups from hatching until they reached adult size. We found that living in groups had a strong (up to 14%) negative effect on growth in fish from low-predation populations, despite the lack of constraints originating from resource limitation, predation, reproduction or parasites. Group living had no effect on the growth of fish from high-predation populations. We also studied willingness to shoal: fish from all populations showed strong shoaling behaviour. Our results suggest that the social cost of shoaling can be high, but individuals from high-predation populations seem to have adapted to minimize these costs better than individuals from low-predation populations.

Group living occurs in many taxa and has attracted considerable attention (e.g. Pitcher & Parrish 1993; Krause & Ruxton 2002). A lot of research has been done to identify its ultimate causes, and several (nonexclusive) benefits of group formation have been discovered. The most obvious benefit of grouping is the decreased per capita predation risk. This can result from increased vigilance in the group (Treherne & Foster 1980; Magurran et al. 1985), dilution of predation risk (Foster & Treherne 1981; Godin 1986) or predator confusion (Landeau & Terborgh 1986; Magurran & Pitcher 1987). These mechanisms can act separately or together. Besides anti-predator advantages, joining a group can increase foraging efficiency (Pitcher et al. 1982; Magurran & Pitcher 1983) and mate-finding success (Höglund & Alatalo 1995) and decrease the prevalence of mobile parasites (Poulin & Fitzgerald 1989; Mooring & Hart 1992) and the energetic costs of movement (Herskin & Steffensen 1998; Weimerskirch et al. 2001).

Even though much more effort has been spent studying the benefits than the costs of grouping (Krause & Ruxton 2002), some areas where the main costs should lie have been identified. The most obvious costs of grouping are those connected to the sharing of limited resources and are expected to manifest themselves as different forms of competition (e.g. Pitcher & Parrish 1993; Krause & Ruxton 2002). In addition, certain predatory strategies act independently of the antipredator function of shoals (Krause et al. 1998), and some predators preferentially attack shoals over singletons (Krause & Godin 1995; Botham & Krause 2005; Ioannou & Krause 2008). Finally, while grouping can be advantageous against mobile and behaviourally flexible parasites thanks to the dilution effect (Poulin & Fitzgerald 1989; Mooring & Hart 1992), there may be considerable costs caused by less mobile, contact-spread parasites (Rubinstein & Hochmann 1989; Côté & Poulin 1995; Poulin 1999).

Perhaps the least studied component of group living is the 'cost of sociality', what we would define as the cost that is independent of the resources actually available and of the distribution of predators or parasites, is not related directly to reproduction, and results merely from the social interactions (e.g. aggression related to dominance hierarchy) among group members, in other words, the costs associated with repeated and/or continued interactions between individuals. If such costs of grouping are relevant, selection should favour behaviours that minimize it, while simultaneously preserving the benefits of group living. This selection

* Correspondence: G. Herczeg, Ecological Genetics Research Unit, Department of Biological and Environmental Sciences, University of Helsinki, P.O. Box 65, FI-00014, Finland.
E-mail address: gabor.herczeg@helsinki.fi (G. Herczeg).

0003-3472/$38.00
doi:10.1016/j.anbehav.2008.10.023

should be especially strong in populations where grouping is an important component of fitness, but less so in populations where the benefits from grouping are lower (i.e. predation pressure is low, food is evenly distributed). One possible mechanism behind this could be the negative covariation of shoaling tendency and aggression (Magurran & Seghers 1991).

The nine-spined stickleback has a circumpolar distribution, and occurs in a wide variety of habitats from large lake systems, rivers and coastal areas to small creeks and ditches. It can also successfully persist in ponds as the only fish species (e.g. Bănărescu & Paepke 2001; Östlund-Nilsson et al. 2007). Among the various biotic and abiotic environmental factors, predation pressure is one that varies greatly across habitat types. Hence, it provides an excellent model to test for geographical variation in the social cost of shoaling.

Our aim in this study was to test whether (1) the cost of sociality was detectable in growth (from hatching to reaching adult size), and (2) populations living under differential predation risk have diverged in their ability to cope with this cost. In addition, we tested for differences in the willingness to shoal between populations. We hypothesized that when the expected fitness benefits from shoaling are large (populations living sympatrically with predatory fish), selection for (1) mechanisms reducing the negative consequences of group living and (2) increased shoaling tendency should also be strong. Consequently, we predicted that the social costs of shoaling should be lower and the willingness to shoal more pronounced among fish originating from populations with a high than alow-predation risk. To test this, we bred *P. pungitius* from four geographically (Fig. 1) and genetically (T. Shikano, G. Herczeg & J. Merilä, unpublished data) isolated populations differing markedly in their predation risk, and reared them either individually or in shoals in a laboratory setting. By measuring their growth, we estimated the fitness consequences of living in shoals for the different populations. We also conducted a behavioural experiment to quantify their willingness to shoal. As far as we are aware, this is the first experiment testing for evolutionary differences in the ability to cope with the social costs of grouping from hatching to reaching adult size.

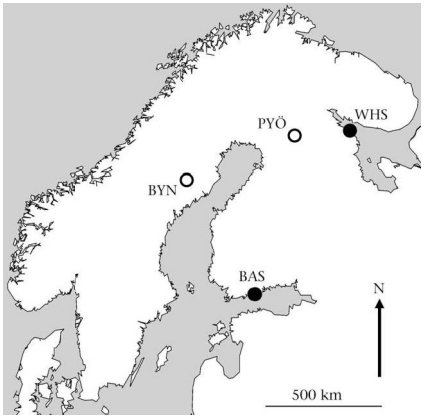


Figure 1. Map of Scandinavia with the sampling localities (filled circles denote marine, open circles pond populations). The sampled populations are Baltic Sea near Helsinki, Finland (BAS), White Sea, Levin Navolok Bay, Russia (WHS), Bynastjärnen, Sweden (BYN) and Pyöreälampi, Finland (PYÖ).

METHODS

Fish Sampling and Rearing

We collected adult sticklebacks from four populations (Fig. 1) before or during the early phase of the reproductive period in 2007. We sampled two ponds (both isolated), Pyöreälampi, Finland, and Bynästjärnen, Sweden, and two marine populations, the Baltic Sea near Helsinki, Finland, and the White Sea in the Levin Navolok Bay, Russia. The four populations are genetically (highly) isolated from each other as revealed by microsatellite markers (T. Shikano, G. Herczeg & J. Merilä; unpublished data). The ponds had a surface area of less than 5 ha and maximal depth around 5–10 m. The nine-spined stickleback is the only fish species in Bynastjärnen, while a few recently introduced whitefish, *Coregonus lavaretus*, occur in Pyöreälampi. Given the lack of predatory fishes in the ponds, mortality from this source is nonexistent among adult sticklebacks in these populations. Avian predation is another potential source of predation-induced mortality but there are no fish-eating birds breeding in the ponds. Hence, apart from the effects of some sporadic visiting birds, bird predation on sticklebacks in ponds is likely to be negligible. For the sampling, we used minnow traps deployed from the shore as well as seine nets. Adult sticklebacks were transported to the aquaculture facilities of the University of Helsinki and kept under 24 h light (characteristic at high latitudes during the breeding season) and at 17 °C. They were fed with frozen bloodworms (*Chironomidae* sp.) until enough individuals from the populations were in reproductive condition.

Five full-sib crosses per population were done during 1 week between 25 June and 1 July. The crosses were done by gently squeezing the eggs out from ripe females, and pouring sperm solution on them. To obtain the sperm solution we humanely killed the males by over anaesthetizing them in MS 222 (0.23 g/litre water solution with 0.46 g $NaHCO_3$/litre water to buffer the PH). To ensure brain death we left the males in the anaesthetic for an extra ca. 10 min after they stopped reacting visibly to physical stimuli. We then dissected and minced the testicles in a drop of water to obtain the sperm. Thirty minutes after fertilization clutches were checked under a dissecting microscope and visibly unfertilized eggs were removed. The clutches were put in 1.4-litre tanks in two Allentown Zebrafish Rack Systems (hereafter 'rack', Aquaneering Inc., San Diego, CA, U.S.A.) and checked daily for unfertilized eggs until hatching. Both racks had a closed water-circulating system with multilevel filtering (physical, chemical, biological and UV filters) and inbuilt thermostats. The water temperature was set to 17 °C and 24 h light was provided.

All clutches hatched 7 days after fertilization (including the day of fertilization) and the fry reached the free-swimming stage 4 days later. At this stage, each family was divided into two treatments (see below) and feeding was initiated. In both treatments, fish were fed twice a day ad libitum (uneaten food had to be removed frequently). Feeding was started with live brine shrimp (*Artemia* sp.) nauplii, and as fish grew, they were provided with frozen copepods (*Cyclops* sp.) and later with frozen bloodworms. Each of the diet switches took 2–3 weeks during which the old and the new food types were provided in parallel. At the time when each of the new food types was provided alone, all fish from the given treatment and population were able to consume them easily. We did not attempt to mimic the natural photoperiod because the different populations were from different latitudes (Fig. 1). We simply changed from 24 h light to a 12:12 h light:dark cycle gradually (over 1 week) after 12 weeks from the beginning of the experiment. Water temperature was kept at 17 °C throughout the experiment. All experiments were carried out in freshwater. Nine-spined sticklebacks live in both seawater and freshwater environments. The marine populations in our study came from coastal

bays. We changed the water of the site of capture to freshwater gradually in the case of the wild-caught marine fish, while the laboratory fish hatched straight into freshwater. No signs of stress were observed.

Treatments and Measurements

We assigned 4-day-old fry to one of two treatments. In the first treatment (hereafter individual treatment), 10 individuals were chosen from each family and reared individually in randomly chosen 1.4-litre tanks in the racks (one rack contained 100 tanks), summing to a total of 200 individually reared fish (four populations × five families × 10 individuals). Visual contact among individual fish was prevented by plastic panels fitted between the tanks. Chemical contact could not be blocked owing to the closed water system of the racks; therefore the two treatments were similar in this respect.

In the second treatment (hereafter shoal treatment), all families (after the 10 fish for the individual treatment were removed from each) were divided into two replicates (maximum of 40 individuals per replicate) and the replicates were placed in 10-litre aerated plastic tanks. After 3–4 weeks, the family replicates were transferred to similar 10-litre tanks with mosquito netting on the sides. These tanks were placed into large plastic tanks (76 × 54 cm and 40 cm highs, eight 10-litre tanks in each) that were equipped with an open, one-way water flow, so that the water was changed through the mosquito net sides of the 10-litre tanks holding the replicates. The 10-litre tanks were placed randomly into the large tanks, but each replicate within a family was placed into a different large tank. After another 3–4 weeks (depending on the day of fertilization), 20 fish per family were chosen (replicates equally represented), and pooled within each population (100 fish per population). From the Baltic population, it was impossible to ensure equal family representation and a sample size of 100 because of the low number of individuals in the original families and subsequent mortality. For this population, we used 93 fish (26, 24, 21, 16 and 6 per family, respectively). Each population pool was divided into two replicates. The population replicates were placed randomly into the halves of large plastic tanks (76 × 54 cm and 40 cm high) divided by mosquito netting and with an open, one-way water flow. The replicates within a population were placed into different tanks. The water volume was set to 140 litres in the large tanks; hence, the per capita water volume (1.4 litres) or, in other words, the fish density, was similar between treatments from here onwards.

Fish were measured 12 and 20 weeks after the treatments started, respectively. We measured all fish from the individual treatment, and a randomly selected 50 fish (25 per replicate) from each population from the shoal treatment. Owing to mortality (of unknown causes but mostly at very young stages), the sample sizes in the individual treatment were as follows: $N = 45$ for Bynastjärnen, $N = 42$ for Pyöreälampi, $N = 47$ for the Baltic Sea and $N = 48$ for the White Sea populations. Digital photographs were taken from the side of the fish in a standardized way with a ruler placed in each photograph for scaling, and standard length (hereafter SL; from the tip of the snout to the end of the tail base) was measured from the images using tpsDig 1.37 (F. J. Rohlf, http://life.bio.sunysb.edu.morph).

Shoaling Experiment

Behavioural experiments were conducted to quantify the willingness to shoal in the different populations between 12 and 14 November (approximately 2 weeks before the second measurement). A randomly chosen sample of 16 fish from each population from the shoal treatment was tested by applying the set-up outlined in Krause & Ruxton (2002) for quantifying shoaling behaviour. In short, we used four aquaria (76 × 34 cm and 34 cm high) with three compartments separated by Plexiglas walls. Chemical contact was possible between the compartments. In one of the side compartments (13 × 34 cm and 34 cm high) we placed six randomly chosen fish from the given populations as a shoal stimulus, and the other empty side compartment served as a control stimulus. The same fish served as shoal stimuli in all experimental trials, and they had a day of acclimation before the experiment. They were fed twice a day (before and after the experiment). The central compartment (50 × 34 cm and 34 cm high) was divided (and marked) into four equal sectors. Sectors were numbered '1' for the closest sector to the shoal, '4' for the closest sector to the control stimulus, and '2' and '3' in between. Water temperature and photoperiod were similar to those in the treatments above. After half of the experimental trials were done (eight fish per population), the stimulus shoals were randomly redistributed among the aquaria and placed into the opposite side to before. Because both focal and stimulus fish were similar in age, and had been kept similarly, we could exclude possible confounding effects of size-assortative shoaling which have been described in stickleback species (e.g. Ranta & Lindström 1990; Ranta et al. 1992).

Experimental fish were placed into a transparent plastic cylinder (12 cm diameter) in the middle of the central compartment and left to acclimate and orient for 5 min. After that, the cylinder was gently lifted by pulling a string and the movements of the fish between the four sectors were recorded in 5 s intervals for 5 min. Observations were made from a blind. Experimental trials were conducted between 0900 and 1700 hours. Each population was represented in all four consecutive experimental trials in a random order.

Data Analysis

To test for the potential effects of the replicates in the shoal treatment, we applied general linear mixed models (GLMMs) with population as a fixed factor, replicate nested within population as a random factor, and SL as the dependent variable. There were no differences between replicates within populations in either of the measurements (week 12: $Z = 1.13$, $P = 0.13$; week 20: $Z = 0.75$, $P = 0.23$). Our design (mixed family pools in the shoal treatment) did not allow us to include the family structure in our analyses, and so the reported degrees of freedom might be viewed as too high, although related individuals are likely to occur in the same shoals in the wild too. However, when we adjusted the degrees of freedom in our analyses to the level of statistically independent samples (i.e. the number of independent families, $N = 5$ per population) all significant effects in the analyses remained significant. Hence, the unadjusted results are reported.

We used GLMMs with habitat (marine versus pond), treatment (individual versus shoal) and their interaction as fixed factors, population nested in habitat as a random factor and SL as the dependent variable separately for the 12 and 20 weeks' measurements. We could not use one model to test for all effects including time of measurement because of the repeated measures nature of data from the individual treatment and the unknown individual overlap in the shoal treatment. However, as the two GLMMs were not independent, we considered significance levels by applying the sequential Bonferroni correction (Rice 1989). The correction did not affect our results. To perform pairwise population comparisons, we ran general linear models (GLMs) with population, treatment and their interaction as fixed factors and SL as the dependent variable separately for the 12 and 20 weeks' measurements, and used Scheffé post hoc tests to compare populations at the pairwise level.

To describe the tendency for shoaling, we calculated an index for each individual as:

$$I = (n_1 + 2n_2 + 3n_3 + 4n_4)/(n_1 + n_2 + n_3 + n_4)$$

where n_1 = the number of times a fish was observed in sector 1, n_2 = the number of times a fish was observed in sector 2, etc. Owing to the non-normal distributions, shoaling indices were compared between populations with a Kruskal–Wallis ANOVA.

For all statistical analyses we used SAS 9.1 (SAS Institute Inc., Cary, NC, U.S.A.).

Ethical Note

Fish used in this experiment were bred in our laboratory. Mortality during the study period was low, and did not exceed the average rate that we have observed in the species in captivity. The experiments were done under licence from the Helsinki University Animal Experimentation Committee. After the study, fish were retained in captivity to be used in other studies.

RESULTS

The first GLMM revealed a habitat-specific treatment effect on body size in 12-week-old fish (habitat: $F_{1,2} = 0.30$, $P = 0.638$; treatment: $F_{1,376} = 53.86$, $P < 0.001$; habitat*treatment: $F_{1,376} = 49.32$, $P < 0.001$). Fish from ponds were smaller in shoals than when kept individually, while there was no effect of treatment in marine fish. The population effect within habitat was nonsignificant ($Z = 1.00$, $P = 0.159$). The first GLM showed a similar pattern at the population level (population: $F_{3,374} = 190.61$, $P < 0.001$; treatment: $F_{1,374} = 56.19$, $P < 0.001$; population*treatment: $F_{3,374} = 22.03$, $P < 0.001$; Fig. 2). Scheffé's post hoc tests revealed that the treatment affected body size only in the pond, but not in the marine, populations (Bynastjärnen: $P < 0.001$; Pyöreälampi: $P < 0.001$; Baltic Sea: $P = 0.937$; White Sea: $P = 0.842$; Fig. 2). Living in shoals resulted in a 7% reduction in the mean size in the Bynastjärnen population, and in a 12% reduction in the Pyöreälampi population (Fig. 2).

The second GLMM on 20-week-old fish revealed similar patterns as the first (habitat: $F_{1,2} = 1.48$, $P = 0.347$; treatment: $F_{1,376} = 72.86$, $P < 0.001$; habitat*treatment: $F_{1,376} = 70.98$, $P < 0.001$): there was a growth deficit from group living in pond fish, but not in marine fish. The population effect within habitat was nonsignificant ($Z = 1.00$, $P = 0.160$). The second GLM revealed a similar pattern at the population level (population: $F_{3,374} = 284.74$, $P < 0.001$; treatment: $F_{1,374} = 82.14$, $P < 0.001$; population*treatment: $F_{3,374} = 41.73$, $P < 0.001$; Fig. 2). Again, treatment affected body size of pond fish, but not that of marine fish (Scheffé tests: Bynastjärnen: $P < 0.001$; Pyöreälampi: $P < 0.001$; Baltic Sea: $P = 0.113$; White Sea: $P = 0.091$; Fig. 2). Living in shoals resulted in a 7% reduction in the mean size of the Bynastjärnen fish, and 14% in the Pyöreälampi fish.

We did not detect any difference in shoaling behaviour between the populations (Kruskal–Wallis ANOVA: $X^2_{3,64} = 4.86$, $P = 0.18$). All fish showed a strong association with the stimulus shoal (Fig. 3).

DISCUSSION

Our results suggest that living in groups can have a considerable cost even when (1) food is not a limiting factor, there are no (2) predators or (3) parasites in the system, and even when (4) the constraints and costs of reproduction are ruled out. Hence, the manifested cost can only be related to sociality itself, resulting purely from contact with conspecifics. Furthermore, we found systematic differences in the presence of such a social cost between different population types: the cost was high in pond populations but statistically undetectable in marine populations. Given that the fish were bred and reared in the same laboratory setting, the observed differences are likely to have a genetic basis. Hence, the systematic differences in costs of shoaling between geographically and genetically isolated populations living under contrasting predation regimes suggests that the pattern is likely to be a result of natural selection (Clarke 1975; Endler 1986).

Apart from direct measures of fitness (see e.g. Jones et al. 2003), growth rate has been used extensively as a proxy of fitness in three-spined sticklebacks, *Gasterosteus aculeatus* (Bolnick & Lau 2008, and references therein). Because female reproductive output (Heins et al. 2005) and most probably male reproductive success are strongly size dependent in *P. pungitius*, the observed growth

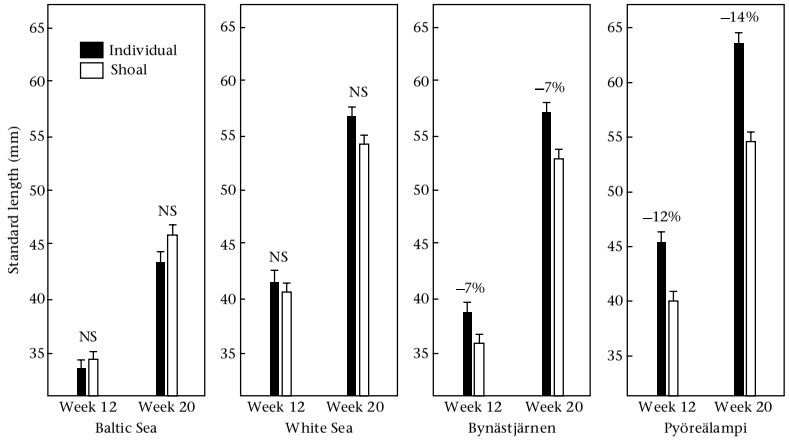


Figure 2. Mean standard length +95% confidence intervals of individually and shoal-reared *Pungitius pungitius* from different populations, measured at 12 and 20 weeks after the free-swimming stage (4 days after hatching). Nonsignificant post hoc comparisons (Scheffé tests) and the percentage of significant differences between individually or shoal-reared fish are shown above the bars.

doi:10.1016/j.anbehav.2008.11.014

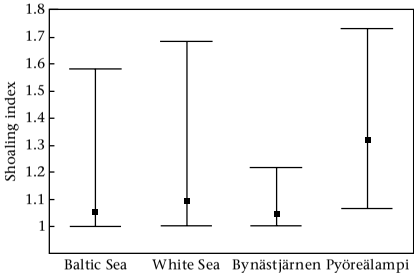


Figure 3. Shoaling behaviour of *Pungitius pungitius* from different populations reared in the shoal treatment. Median shoaling index (for details see text) ± quartiles are shown. An index of 1 translates to perfect shoaling, 4 to perfect shoal avoidance, while a median around 2 would mean random spatial distribution.

reduction stemming from shoaling is likely to translate to reduced fitness in this species too. We found that growth of pond population individuals was strongly constrained by the shoal treatment, resulting in 7 and 14% growth reduction in 20 weeks in Bynastjärnen and Pyöreälampi fish, respectively. It is noteworthy that during the 20 weeks, fish in our experiment had reached mature size (according to e.g. Bănărescu & Paepke 2001). Hence, the observed growth deficiency might represent a considerable fitness cost. This might be especially relevant in the pond populations where fish are larger, and grow faster and for longer, than the fish from marine environments (G. Herczeg, A. Gonda & J. Merilä, unpublished data). Furthermore, pond fish live longer and are more aggressive and bolder than the fish from larger water systems, possibly as a result of selection stemming from the increased intraspecific competition in the absence of predation and interspecific competition (Herczeg et al., in press; G. Herczeg, A. Gonda & J. Merilä; unpublished data). Somewhat surprisingly, we did not find a positive effect of shoaling on growth in the marine populations, even though being solitary for an individual with a drive towards shoaling has shown to result in lowered growth rates (Peuhkuri et al. 1995; Strand et al. 2007).

The choice of an individual to join a shoal is based on constant reappraisal of the benefits and costs of being social (Pitcher & Parrish 1993). The most important benefits for teleost fishes are probably predator avoidance, increased foraging success for aggregated food patches, and the reduced costs of travelling in open habitats (Magurran 1990a; Ranta & Kaitala 1991; Pitcher & Parrish 1993; Krause & Ruxton 2002). However, these benefits may vary among different habitats (e.g. Brown & Warburton 1997). Competition and the resulting aggression is expected to be costly and elevated in shoals, but selection might favour behaviours minimizing conflict where the benefits of sociality are high (Giraldeau & Caraco 2000). Fish in the Baltic or White Seas should face various fish predators, and, thus, they might enjoy the benefits of shoaling. On the other hand, fish from the ponds are living under low-predation pressure, and, hence, shoaling as an antipredator defence might be meaningless for them. Predatory insects, leeches and sporadic avian predators are potential predators in the ponds, but shoaling is probably not an effective means of avoiding mortality by these predators. We have no information about the distribution of food in the different habitats, but we would expect food distribution to be patchier in marine than in pond environments. All in all, shoaling seems to be beneficial in marine environments, but unimportant in ponds, and consequently marine populations are expected to evolve to minimize the social costs of shoaling, while pond populations are not. Furthermore, it is assumed that sexual selection favouring larger size (e.g. Andersson 1994) results in higher growth rates under low-predation pressure (Blanckenhorn 2000), and higher growth is coupled with higher energy demands and higher aggression levels as a consequence. Thus, this chain of logic can also lead us to the prediction that pond fish have a high social cost of shoaling. This prediction is fully supported by our results: marine populations did not encounter growth costs in shoals, whereas pond populations were 7–14% smaller after 20 weeks in the shoal than in the individual treatment.

The idea that differences in selective environments can result in considerable geographical variation in behaviour is well established (Foster & Endler 1999). For instance, differences in predatory pressure can result in a difference in boldness (Brown et al. 2005) and such a difference can be heritable (Brown et al. 2007). A tendency for shoaling is also dependent on predation risk (Brown & Warburton 1997) and is heritable (Breden et al. 1987; Magurran 1990b). A series of experiments on wild guppies, *Poecilia reticulata*, revealed (co) variation in shoaling, aggression and predator inspection behaviours between populations differing in predation pressure (e.g. Magurran & Seghers 1991, 1994). Hence, especially in the light of our results about the different costs of shoaling, it is surprising that we did not find any differences in shoaling tendency between our populations; all fish spent most of their time shoaling. However, if we assume that time spent under stress (and thus shoaling) differs between high- and low-predation populations in the wild, selection should still favour lower aggression levels (and thus lower social costs of shoaling) in high-predation populations. It is noteworthy that our set-up adopted to study shoaling tendency (following e.g. Krause & Ruxton 2002) tested for shoaling tendency under stressful (experimental fish were introduced to a new environment), rather than under 'ordinary' conditions. Also, our relatively simple tests might not have been sensitive enough to detect small population differences. Other types of tests, based for example on interindividual distances in a nonstressed situation, might provide stronger tests in our system as well. In theory, there are several other possible explanations for the lack of difference that we could not test for, such as seasonality or the age (i.e. developmental stage) of the fish used in the experiments.

In summary, we found support for the existence of a social cost of grouping that was independent of resources, predators, parasites or reproduction in first-generation, laboratory-reared *P. pungitius*. Furthermore, the cost was pronounced in fish from two geographically distant populations inhabiting ponds lacking predatory fish, but missing from two geographically separated marine populations subject to predation. These results suggest that the ability to minimize the social cost of grouping had a genetic component, and the systematic differences between population types suggest that the reported pattern is a result of selection. At least two nonexclusive mechanisms are conceivable: (1) natural selection favouring lowered levels of costly social interactions in populations where the benefits of, and the expected time spent with, shoaling are high and (2) sexual selection resulting in larger body size, higher growth rate, higher energy demands and, as a consequence, a higher level of aggression under low-predation pressure.

Acknowledgments

We thank Victor Berger, Göran Englund, Tuomas Leinonen, Daniel Lussetti and Pirkko Siikamäki for helping us in organizing and executing the field sampling. Special thanks to the Oulanka Research Station and the White Sea Biological Station for their help. We are highly indebted to Esa Ranta and two anonymous referees for their constructive comments and John Loehr for correcting the English. We were supported by the Academy of Finland and Centre for International Mobility.

References

Andersson, M. 1994. *Sexual Selection*. Princeton, New Jersey: Princeton University Press.

Bănărescu, P. M. & Paepke, H.-J. 2001. *The Freshwater Fishes of Europe. Vol. 5/III*. Wiebelsheim: AULA-Verlag.

Blanckenhorn, W. U. 2000. The evolution of body size: what keeps organisms small? *The Quarterly Review of Biology*, **75**, 385–407.

Bolnick, D. I. & Lau, O. L. 2008. Predictable patterns of disruptive selection in stickleback in postglacial lakes. *Evolution*, **172**, 1–11.

Botham, M. S. & Krause, J. 2005. Shoals receive more attacks from the wolf-fish (*Hoplias malabaricus* Bloch, 1794). *Ethology*, **111**, 881–890.

Breden, F., Scott, M. A. & Michel, E. 1987. Genetic differentiation for anti-predator behaviour in the Trinidad guppy, *Poecilia reticulata*. *Animal Behaviour*, **35**, 618–620.

Brown, C. & Warburton, K. 1997. Predation recognition and anti-predator responses in the rainbowfish *Melanotaenia eachamensis*. *Behavioral Ecology and Sociobiology*, **41**, 61–68.

Brown, C., Jones, F. & Braithwaite, V. A. 2005. In situ examination of boldness–shyness traits in the tropical poeciliid, *Brachyraphis episcopi*. *Animal Behaviour*, **70**, 1003–1009.

Brown, C., Burgess, F. & Braithwaite, V. A. 2007. Heritable and experiential effects on boldness in a tropical poeciliid. *Behavioral Ecology and Sociobiology*, **62**, 237–243.

Clarke, B. C. 1975. The contribution of ecological genetics to evolutionary theory: detecting the direct effects of natural selection on particularly polymorphic loci. *Genetics, Supplement*, **79**, 101–113.

Côté, I. M. & Poulin, R. 1995. Parasitism and group size in social animals: a meta-analysis. *Behavioral Ecology*, **6**, 159–165.

Endler, J. A. 1986. *Natural Selection in the Wild*. Princeton, New Jersey: Princeton University Press.

Foster, S. A. & Endler, J. A. 1999. *Geographic Variation in Behavior: Perspectives on Evolutionary Mechanisms*. New York: Oxford University Press.

Foster, W. A. & Treherne, J. E. 1981. Evidence for the dilution effect in the selfish herd from fish predation on a marine insect. *Nature*, **293**, 466–477.

Giraldeau, L.-A. & Caraco, T. 2000. *Social Foraging Theory*. Princeton, New Jersey: Princeton University Press.

Godin, J.-G. J. 1986. Risk of predation and foraging behaviour in shoaling banded killifish (*Fundulus diaphanus*). *Canadian Journal of Zoology*, **64**, 1675–1678.

Heins, D. C., Baker, J. A., de Silva, G. & Birden, E. L. 2005. Clutch characteristics of two populations of nine-spined stickleback from south-central Alaska. *Journal of Fish Biology*, **67**, 873–878.

Herczeg, G., Gonda, A. & Merilä, J. In press. Predation mediated population divergence in complex behaviour of ninespine stickleback *(Pungitius pungitius)*. *Journal of Evolutionary Biology*, doi: 10.1111/j.1420-9101.2008.01674.x.

Herskin, J. & Steffensen, J. F. 1998. Energy savings in sea bass swimming in a school: measurements of tail beat frequency and oxygen consumption at different swimming speeds. *Journal of Fish Biology*, **53**, 366–376.

Höglund, J. & Alatalo, R. V. 1995. *Leks*. Princeton, New Jersey: Princeton University Press.

Ioannou, C. C. & Krause, J. 2008. Searching for prey: the effects of group size and number. *Animal Behaviour*, **75**, 1383–1388.

Jones, F. C., Brown, C. & Braithwaite, V. A. 2008. Lack of assortative mating between incipient species of stickleback from a hybrid zone. *Behaviour*, **145**, 463–484.

Krause, J. & Godin, J.-G. J. 1995. Predator preferences for attacking particular prey group sizes: consequences for predator hunting success and prey predation risk. *Animal Behaviour*, **50**, 465–473.

Krause, J. & Ruxton, G. D. 2002. *Living in Groups*. Oxford: Oxford University Press.

Krause, J., Ruxton, G. D. & Rubenstein, D. 1998. Is there always an influence of shoal size on predator hunting success? *Journal of Fish Biology*, **52**, 494–501.

Landeau, L. & Terborgh, J. 1986. Oddity and the confusion effect in predation. *Animal Behaviour*, **34**, 1372–1380.

Magurran, A. E. 1990a. The adaptive significance of schooling as an anti-predator defence in fish. *Annales Zoologici Fennici*, **27**, 51–66.

Magurran, A. E. 1990b. The inheritance and development of minnow anti-predator behaviour. *Animal Behaviour*, **39**, 834–842.

Magurran, A. E. & Pitcher, T. J. 1983. Foraging, timidity and shoal size in minnows and goldfish. *Behavioral Ecology and Sociobiology*, **12**, 142–152.

Magurran, A. E. & Pitcher, T. J. 1987. Provenance, shoal size and the sociobiology of predator-evasion behaviour in minnow shoals. *Proceedings of the Royal Society of London, Series B*, **229**, 439–465.

Magurran, A. E. & Seghers, B. H. 1991. Variation in schooling and aggression amongst guppy (*Poecilia reticulata*) populations in Trinidad. *Behaviour*, **118**, 214–234.

Magurran, A. E. & Seghers, B. H. 1994. Predator inspection behaviour covaries with schooling tendency amongst wild guppy, *Poecilia reticulata*. *Behaviour*, **128**, 121–134.

Magurran, A. E., Oulton, W. J. & Pitcher, T. J. 1985. Vigilant behaviour and shoal size in minnows. *Zeitschrift für Tierpsychologie*, **67**, 167–178.

Mooring, M. S. & Hart, B. J. 1992. Animal grouping for protection from parasites: selfish-herd and encounter-dilution effects. *Behaviour*, **123**, 173–193.

Östlund-Nilsson, S., Mayer, I. & Huntingford, F. A. 2007. *Biology of the Three-spined Stickleback*. Boca Raton: CRC Press.

Peuhkuri, N., Ranta, E., Juvonen, S. K. & Lindström, K. 1995. Schooling affects growth in the 3-spined stickleback (*Gasterosteus aculeatus*). *Journal of Fish Biology*, **46**, 221–226.

Pitcher, T. J. & Parrish, J. K. 1993. Functions of shoaling behaviour in teleosts. In: *Behaviour of Teleost Fishes* (Ed. by T. J. Pitcher), pp. 363–439, 2nd edn. London: Chapman & Hall.

Pitcher, T. J., Magurran, A. E. & Winfield, I. J. 1982. Fish in larger shoals find food faster. *Behavioral Ecology and Sociobiology*, **10**, 149–151.

Poulin, R. 1999. Parasitism and shoal size in juvenile sticklebacks: conflicting selection pressures from different ectoparasites. *Ethology*, **105**, 959–968.

Poulin, R. & Fitzgerald, G. J. 1989. Shoaling as an anti-ectoparasite mechanism in juvenile sticklebacks (*Gasterosteus* ssp.). *Behavioral Ecology and Sociobiology*, **24**, 251–255.

Ranta, E. & Kaitala, V. 1991. School size affects individual feeding success in three-spined sticklebacks (*Gasterosteus aculeatus* L.). *Journal of Fish Biology*, **39**, 733–737.

Ranta, E. & Lindström, K. 1990. Assortative schooling in three-spined stickleback? *Annales Zoologici Fennici*, **27**, 67–75.

Ranta, E., Juvonen, S.-K. & Peuhkuri, N. 1992. Further evidence for size-assortative schooling in sticklebacks. *Journal of Fish Biology*, **41**, 627–630.

Rice, W. R. 1989. Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.

Rubinstein, D. I. & Hochmann, M. E. 1989. Parasites and social behaviour of island feral horses. *Oikos*, **55**, 312–320.

Strand, Å, Alanärä, A. & Magnhagen, C. 2007. Effect of group size on feed intake, growth and feed efficiency of juvenile perch. *Journal of Fish Biology*, **71**, 615–619.

Treherne, J. E. & Foster, W. A. 1980. The effect of group size on predator avoidance in a marine insect. *Animal Behaviour*, **28**, 1119–1122.

Weimerskirch, H., Martin, J., Clerquin, Y., Alexandre, P. & Jiraskova, S. 2001. Energy saving in flight formation. *Nature*, **413**, 697–698.

Journal of Animal Ecology

Journal of Animal Ecology 2010, **79**, 581–588 doi: 10.1111/j.1365-2656.2010.01665.x

Rensch's rule inverted – female-driven gigantism in nine-spined stickleback *Pungitius pungitius*

Gábor Herczeg*, Abigél Gonda and Juha Merilä

Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, P.O. Box 65, FI-00014, Helsinki, Finland

Summary

1. Allometric scaling of sexual size dimorphism (SSD) with body size is a commonplace occurrence in intraspecific or interspecific comparisons. Typically, SSD increases with body size when males, and decreases when females are the larger sex – a pattern known as Rensch's rule. Intraspecific studies of Rensch's rule in vertebrates are extremely scarce.

2. In an allometric SSD–body size relationship, the sex with the larger body size variation is the driver of size divergence whereas the other sex is following it owing to correlational selection. Hence, one can test which sex is responsible for the observed body size divergence within this framework.

3. Nine-spined stickleback (*Pungitius pungitius*) provides an excellent model to study intraspecific variation in SSD owing to the large interpopulation variation in mean body size. Using data on body size variation in 11 nine-spined stickleback populations covering the full known size range of the species, we investigated: (i) whether variation in SSD scales allometrically with mean body size across the populations; (ii) which sex is driving the allometric relationship and (iii) whether the observed pattern is likely to have a genetic component. In addition, we analysed the size dependency of female reproductive output.

4. We found strong support for an inverse of Rensch's rule: level of female-biased SSD increased with increasing mean size while females were the more variable sex. Results from a common garden experiment supported the pattern found in the wild. Females from giant populations had 2–3 times larger reproductive output than normal-sized females.

5. The fact that females were the more variable sex indicates that the evolution of gigantism in nine-spined sticklebacks is driven by females, and the 2–3 times larger reproductive output per clutch of giant vs. normal-sized females suggests fecundity selection to have an important role in it. Our results oppose the commonly held view that males drive the evolution of SSD as a result of sexual selection favouring larger males.

Key-words: allometry, body size, fecundity selection, geographic variation, sexual selection, sexual size dimorphism

Introduction

Body size is an ecologically and evolutionary important trait, which influences and often correlates with a number of physiological and fitness traits both within and among populations and species (Peters 1983; Roff 1992; Stearns 1992). Interpopulation variation in body size can result from several co-occurring evolutionary and ecological factors. Apart from random events (Wasserzug *et al*. 1979), prey size distribution, predation risk, resource levels and the degree of interspecific and intraspecific competition can all contribute to body size evolution (e.g. Wilson 1975; Case 1978, 1979; Gittleman 1985; Blanckenhorn 2000; Simberloff *et al*. 2000; Clegg & Owens 2002; Boback 2003; Wu, Li & Murray 2006).

Body size can also differ considerably between sexes of the same species (or population), a phenomenon known as sexual size dimorphism (SSD). SSD is common among both plants and animals (Fairbairn 1997), but whether females or males are the larger sex varies in taxon specific manner. In invertebrates and ectothermic vertebrates females are typically the larger sex, whereas in endothermic animals, males are generally larger than females (Fairbairn & Preziosi 1994; Fairbairn 1997; Blanckenhorn 2005; and references therein). Furthermore, the degree of SSD appears to correlate with the mean body size of the species (or population) displaying an allometric relationship (e.g. Fairbairn 1997). In species where females are the larger sex, increasing size is coupled with decreasing SSD (hypo-allometry), whereas in species where males are the larger sex, SSD increases with increasing mean size (hyper-allometry). This relationship is known as the Rensch's rule (e.g. Rensch 1950, 1959; Fairbairn 1997). Note that hypo- and hyper-allometry represent the two 'ends' of one relationship where males are the more variable sex (Δ male size > Δ female size; see fig. 1 in Fairbairn & Preziosi 1994 or Fairbairn 1997). In general, this common allometry implies that: (i) males display larger evolutionary size divergence than females, and that (ii) there is a strong covariance between male and female size. Even though allometry in male-biased SSD follows Rensch's rule in the majority of the studied taxa (e.g. Fairbairn 1997; Colwell 2000; Kratochwil & Frynta 2002; Székely, Freckleton & Reynolds 2004; Johansson, Crowley & Brodin 2005; Fairbairn, Blanckenhorn & Székely 2007), there are some rare exceptions showing an inverse of Rensch's rule (cf. Fairbairn 1997) and the trend is questionable in taxa with female-biased SSD (Webb & Freckleton 2007; Stephens & Wiens 2009). However, the correlational selection hypothesis evoked by Fairbairn (1997) accommodates both trends, stating that ''…allometry for SSD evolves as a consequence of directional selection acting primarily on one sex (e.g. sexual selection on males or fecundity selection on females) combined with correlational selection on the other sex''. Hence, by studying allometry in SSD, one should be able to test which of the sexes is driving body size evolution in a given study system/organism. In other words, given the correlational evolution of body size between sexes, the sex showing greater variation among species/populations should be the driver. Rensch's rule was originally formulated at the interspecific level and most of its large scale tests are performed between species and within lineages (Abouheif & Fairbairn 1997; Székely *et al*. 2004; Johansson *et al*. 2005; Serrano-Meneses *et al*. 2009; Stephens & Wiens 2009) whereas intraspecific studies in vertebrates are scarce.

Gigantism has been observed in a few cases in the three-spined stickleback (*Gasterosteus aculeatus* Linneaus) and explained as an evolutionary response to the presence of gape-limited predators (Moodie 1972a, b; Moodie & Reimchen 1976; Bell 1984; Reimchen 1988, 1991). Gigantism has also been found in the nine-spined stickleback, *Pungitius pungitius* Linneaus (Kuusela 2006; Merilä 2006). We have demonstrated elsewhere that *P. pungitius* living in geographically and genetically isolated small ponds, being released from the constraints of predation and interspecific competition, are larger – sometimes reaching giant sizes – than their conspecifics in lake or sea populations where they coexist with several predatory and competing fish (Herczeg, Gonda & Merilä 2009a).

In this study, we provide one of the first (also see Lengkeek *et al*. 2008) intraspecific tests of Rensch's rule in fish, using data on SSD from 11 (Fig. 1 and Table 1) nine-spined stickleback populations differing markedly in their mean body size and covering the whole known size range of the species. Data from a common garden experiment using a subset of the populations were utilized to demonstrate that the observed patterns were not attributable to only phenotypic plasticity, but had a genetic component. The Rensch's rule framework was also used to test which sex is driving gigantism in *P. pungitius*. By analysing data on female reproductive output in different populations, we also evaluated the plausibility of the hypothesis that fecundity selection acting on females might be an important explanatory factor for gigantism, and/or the observed patterns population divergence in SSD.

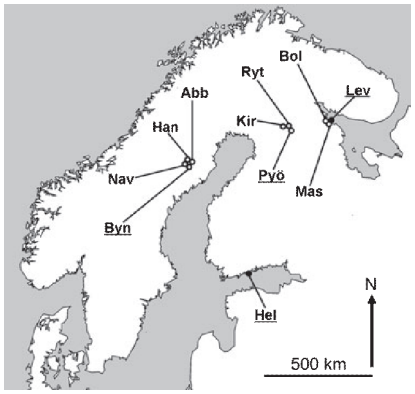


Fig. 1. Map of Fennoscandia showing the location of the sampling sites. Open circles denote small isolated ponds and filled circles marine populations. The underlined populations are the ones represented in the common garden experiment. For population abbreviations, see Table 1.

Materials and methods

SAMPLING SITES AND SAMPLING

Adult fish (N_{male} = 332; N_{female} = 492) were collected from 11 Fennoscandian populations (two marine and nine pond populations; Fig. 1 and Table 1) during the breeding seasons (late May–early July) of 2006–2007. Only samples with enough fish from both sexes in a given year were used (mean $N_{sex\ per\ population}$ = 37·45, 95% CI = 31–44, min–max = 18–81). As *P. pungitius* starts reproduction after its first wintering, and most of its growth takes place before that (Jones & Hynes 1950; Bănărescu & Paepke 2001), distinguishing adults and juveniles is easy. While the Russian populations (Bolotnoje, Mashinnoje and White Sea at Levin Navolok Bay) where the isolation of the ponds happened only recently from the White Sea (Ziuganov & Zotin 1995; information from the White Sea Biological Station) form a genetically indistinguishable group, all other populations were found to be genetically (highly) isolated from each other and from the Russian populations based on analysis of variability of highly polymorphic microsatellite loci (Shikano *et al*. 2010).

Collected fish were killed with an overdose of MS 222 (tricaine methanesulphonate) at the site of capture and stored in 96% ethanol for *c*. 2 months. After this, all individuals were moved to 4% formalin. Standard length (from the tip of the mouth to the end of the tail

*Correspondence author. E-mail: gabor.herczeg@helsinki.fi

Table 1. Sampling sites, their abbreviations, coordinates, sample sizes (males/females) and country. Coordinates of the Russian sites (LEV, BOL, MAS) are approximate (based on Ziuganov & Zotin 1995)

Sampling site	Abbrevations	Sample size	Coordinates	Country
Marine (coastal) populations				
Helsinki, Baltic Sea	HEL	34/37	60°13′ N; 25°11′ E	Finland
Levin Navolok, White Sea	LEV	26/49	66°18′ N; 33°25′ E	Russia
Ponds				
Bolotnoje	BOL	18/25	66°18′ N; 33°25′ E	Russia
Mashinnoje	MAS	23/40	66°18′ N; 33°25′ E	Russia
Pyöreälampi	PYÖ	36/81	66°15′ N; 29°26′ E	Finland
Rytilampi	RYT	19/67	66°23′ N; 29°19′ E	Finland
Kirkasvetinenlampi	KIR	29/44	66°26′ N; 29°08′ E	Finland
Abbortjärn	ABB	40/40	64°29′ N; 19°26′ E	Sweden
Bynästjärnen	BYN	45/46	64°27′ N; 19°26′ E	Sweden
Hansmyrtjärn	HAN	30/31	64°33′ N; 19°10′ E	Sweden
Lill-Navartjärn	NAV	32/32	64°33′ N; 19°11′ E	Sweden

base) was measured at this time with a digital calliper (to an accuracy of 0·01 mm). Gender was identified by eye: male *P. pungitius* develop conspicuous black nuptial colouration during the reproductive season, which becomes even more apparent during the application of MS 222. We note that storage in alcohol can cause some shrinkage. However, length in alcohol is known to become stable in less than 2 months (e.g. Fox 1996; Kristoffersen & Salvanes 1998), and the change found in fish species comparable with *P. pungitius* in size is minor (less than 3%; Kristoffersen & Salvanes 1998).

COMMON GARDEN EXPERIMENT

To assess whether patterns observed in the wild have a genetic component, individuals from four populations (Fig. 1) differing in mean size in the wild (Herczeg, Gonda & Merilä 2009c; Herczeg *et al.* 2009a) were raised in a common garden experiment. We used two geographically isolated ponds (Bynästjärnen and Pyöreälampi, separated by > 500 km) and two marine populations (Baltic Sea near Helsinki and White Sea at Levin Navolok Bay, separated by several thousands of kilometres by coastline distance). Detailed descriptions of the common garden procedures are available from Herczeg, Gonda & Merilä (2009b), Herczeg *et al.* (2009c) and Gonda, Herczeg & Merilä (2009a, b). In short, for each population, five full sib families were created *in vitro* and 10 randomly chosen individuals from each family (4 populations × 5 families × 10 individuals = 200 fish) were transported individually to 1·4-L tanks of two Allentown Zebrafish Rack Systems (Aquaneering Inc., San Diego, USA). The racks were equipped with physical, chemical, biological and UV filters. Temperature was set to 17 °C throughout the experiment. Fish were fed two times a day in excess, first with brine shrimp (*Artemia salina*) nauplii, then with frozen copepods (*Cyclops* sp.) and frozen bloodworms (Chironomidae larvae). As a result of the latitudinal differences between the source populations we did not aim to mimic natural photoperiod changes precisely: we started with a 24 h light period (representative of high latitudes at summer) and changed it to a 12:12 h light–dark periodism gradually during 1 week after 12 weeks.

We aimed to quantify female reproductive output at two stages. First, during the *in vitro* fertilizations we took *c.* 15–20 eggs from each female to 4% formalin. Later five eggs were chosen (randomly from the round-shaped ones) and photographed with a digital camera through a connected dissecting microscope (Wild M5A, Heerbrugg, Switzerland). A millimetre scale was positioned in each photograph for the later measurements. We also counted the total number of eggs in each clutch. Second, the 10 randomly chosen fish used in the common garden experiment from each family were photographed 4 days after hatching when their yolk sac had been absorbed and fish started to swim freely. There was no external feeding done until this stage. Ten individuals had to be discarded either because of early mortality or deformations. Photographs were taken under standardized conditions with a millimetre scale placed in each photograph for scaling. Diameter of the eggs and total length of the fry was measured using tpsDig 1·37 (Rohlf 2002) software.

Finally, common garden fish were measured at the age of 36 weeks [the growth curves of every population and both sexes (i.e. all individuals) had approached their asymptotes at this time; Herczeg, Gonda & Merilä, unpublished data]. Standard length was measured with tpsDig 1·37 (Rohlf 2002) software on digital photographs made from a standard angle from all individuals (a millimetre scale was placed in all photographs as a reference). Body weight was measured with a digital balance to the nearest 0·01 g. Genders were identified via dissection. Individuals from Bynästjärnen could not be analysed as our samples included only two males, so only the remaining three populations were retained for analyses. Due to mortality and other scientific purposes (Gonda *et al.* 2009a, b, unpublished data), we could only use 66 individuals: 21 from the Baltic Sea (family representations: 6, 5, 4, 3, 3), 23 from the White Sea (family representations: 7, 6, 5, 3, 2) and 22 from Pyöreälampi (family representations: 6, 5, 5, 5, 1). We assumed that these individuals are representative for their source populations.

STATISTICAL ANALYSES

First, to analyse the data collected from wild-caught fish, we applied a General Linear Model (GLM) with standard length as dependent variable, and population and sex as fixed factors to test for sex differences in mean standard length among the 11 populations. To test for allometry vs. isometry, the log size of one sex was regressed against the log size of the other using the population means. As in this case neither males' or females' size measurements are fixed (and neither of them is dependent nor independent), and both are measured with error, the standard ordinary least squares (Model I) regression would be statistically incorrect, and major axis regression (Model II) is regarded as more appropriate (e.g. Fairbairn 1997). We run both models for comparative reasons. Following Fairbairn (1997), we placed female size on the *x*-axis. We note that some authors recommend using the maximal values instead of population means (e.g. Stamps & Andrews 1992; Kratochwíl & Frynta 2002). We rerun our GLM using the five largest individuals from each sex in every population and also the regression using the maximal value from each sex in every population, and the results were qualitatively the same as in the analyses based on means (data not shown).

Size of the 36-week-old common garden fish was analysed by General Linear Mixed Models (GLMMs), with body weight or standard length as dependent variables, population and sex as fixed factors and family nested within population as random factor. Size of the females used in the common garden fertilizations was compared with a GLM with population as fixed factor. To test for maternal size dependence in egg volume (calculated from the diameter), egg number and fry size we run GLMMs with mean egg volume (per mother), egg number and mean fry length (per mother) as dependent variables, standard length of the mother as continuous predictor and population as random factor.

Population × sex interactions were included in the models wherever applicable. For the pairwise comparisons following GLMMs, we applied Tukey's *post hoc* tests. Model II regression was done manually following Sokal & Rohlf (1981), while the other analyses were performed with STATISTICA 8·0 (StatSoft. Inc., Tulsa, Oklahoma, USA) and SAS 9·1 (SAS Institute Inc., Cary, NC, USA) for Windows.

Results

SSD IN THE WILD

The GLM revealed that the degree of SSD differed among populations (population: $F_{10,\ 802} = 196{\cdot}72$, $P < 0{\cdot}0001$; sex: $F_{1,\ 802} = 67{\cdot}90$, $P < 0{\cdot}0001$; population × sex: $F_{10,\ 802} = 8{\cdot}00$, $P < 0{\cdot}0001$). Tukey's *post hoc* tests revealed that female-biased SSD was significant in the four populations with the largest mean size (all $P < 0{\cdot}004$; Fig. 2a) whereas in the others no statistical support for the existence of SSD was found (all $P > 0{\cdot}49$; Fig. 2a). Model I regression revealed a significant relationship between the mean size of the sexes across populations and showed that the relationship was significantly different from that assumed under isometry (i.e. both $\beta = 0$ and $\beta = 1$ are rejected; $R^2 = 0{\cdot}97$, $\beta = 0{\cdot}738$, SE(β) = 0·040, $P < 0{\cdot}0001$; Fig. 2b). Model II regression retrieved virtually the same slope ($\beta = 0{\cdot}745$, SE(β) = 0·037). Hence, the results provide strong support for an inverse of Rensch's rule: the detected trend represents hyper-allometry with female-biased SSD.

SSD IN COMMON GARDEN

The GLMMs on the 36-week-old common garden fish revealed that sex differences were population dependent both in standard length (population: $F_{2,\ 11{\cdot}7} = 187{\cdot}44$, $P < 0{\cdot}0001$; sex: $F_{1,\ 63{\cdot}9} = 127{\cdot}16$, $P < 0{\cdot}0001$; population × sex: $F_{2,\ 64} = 12{\cdot}98$, $P < 0{\cdot}0001$; family [population]: $Z = 1{\cdot}68$, $P = 0{\cdot}05$) and body weight (population: $F_{2,\ 9{\cdot}95} = 259{\cdot}60$, $P < 0{\cdot}0001$; sex: $F_{1,\ 55{\cdot}4} = 66{\cdot}35$, $P < 0{\cdot}0001$; population × sex: $F_{2,\ 55{\cdot}9} = 19{\cdot}62$, $P < 0{\cdot}0001$, family[population]: $Z = 0{\cdot}83$, $P = 0{\cdot}20$). Tukey's *post hoc* tests revealed that SSD in standard length was significant in every population ($P < 0{\cdot}02$; Fig. 3a). However, SSD in body weight was only significant in the population with the largest mean size ($P < 0{\cdot}0001$; Fig. 3b), but not in the two smaller populations (all $P > 0{\cdot}14$; Fig. 3b).

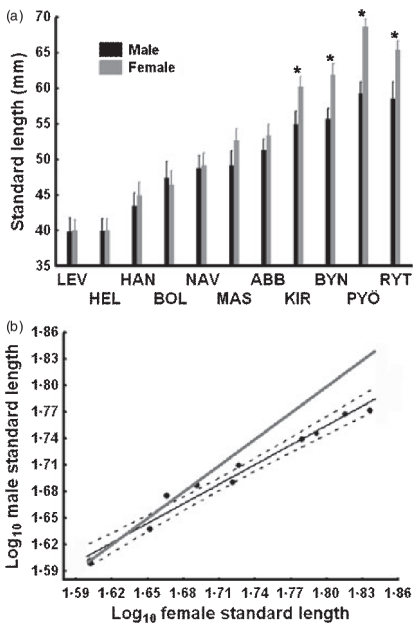


Fig. 2. (a) Sexual size dimorphism across populations measured in wild *Pungitius pungitius* (means + 95% confidence intervals). Asterisks denote populations where *post hoc* Tukey's tests revealed significant sexual differences. (b) Allometry of sexual size dimorphism. Linear (Model I) regression line ($\beta = 0{\cdot}738$) with 95% confidence interval (dotted line) is shown. The thick grey line represents isometry, i.e. $\beta = 1$. For the population abbreviations, see Table 1.

FEMALE REPRODUCTIVE OUTPUT

Standard length of females used for the common garden experiment differed significantly between populations (population: $F_{3,\ 16} = 72{\cdot}33$, $P < 0{\cdot}0001$; mean ± SD; Baltic Sea = 43·39 ± 2·48; White Sea = 44·03 ± 1·85; Bynästjärnen = 62·97 ± 0·75; Pyöreälampi = 74·31 ± 7·29). The number of eggs in a clutch was positively related to the size of the female ($F_{1,\ 4{\cdot}3} = 21{\cdot}54$, $P = 0{\cdot}008$; Fig. 4a). Mean egg volume was unrelated to the size of the mother ($F_{1,\ 6{\cdot}35} = 3{\cdot}15$, $P = 0{\cdot}12$; Fig. 4b). Mean fry size was positively related to the mothers' size ($F_{1,\ 13{\cdot}35} = 13{\cdot}35$, $P = 0{\cdot}04$, Fig. 4c). The population effect was always non-significant ($Z < 0{\cdot}91$, $P > 0{\cdot}18$).

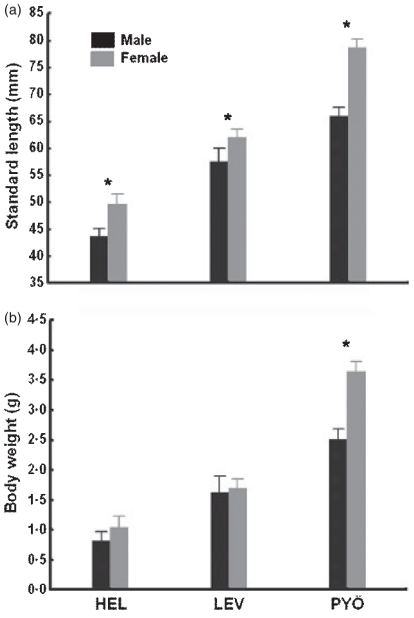


Fig. 3. Sexual size dimorphism (a, standard length; b, body weight) across populations measured in common garden-reared *Pungitius pungitius* (means + 95% confidence intervals are shown). Asterisks denote populations where *post hoc* Tukey's tests revealed significant sexual differences. HEL denotes Baltic Sea near Helsinki; LEV denotes White Sea at Levin Navolok Bay; and PYÖ denotes Pyöreälampi (Finnish pond).

Discussion

The most salient finding of this study is the demonstration of a clear pattern showing the inverse of Rensch's rule in *P. pungitius*. In other words, female-biased SSD displays a hyper-allometric relationship with the mean body size such that SSD increases with increasing mean body size. Several large meta-analyses have found strong support for Rensch's rule whereas only a very few studies have demonstrated an inverse of Rensch's rule (Abouheif & Fairbairn 1997; Fairbairn 1997; Colwell 2000; Lindenfors, Székely & Reynolds 2003; Székely *et al*. 2004; Johansson *et al*. 2005). The majority of studies so far found that body size/SSD evolution is mainly driven by males, resulting from sexual selection favouring larger males (e.g. Berry & Shine 1980; Fairbairn & Preziosi 1994; Abouheif & Fairbairn 1997; Cox, Kelly & John-Adler 2003; Székely *et al*. 2004; Dale *et al*. 2007; Lislevand, Figuerola & Székely 2009; Serrano-Meneses *et al*. 2009), whereas female body size evolves as correlated response to selection on males (Fairbairn 1997).

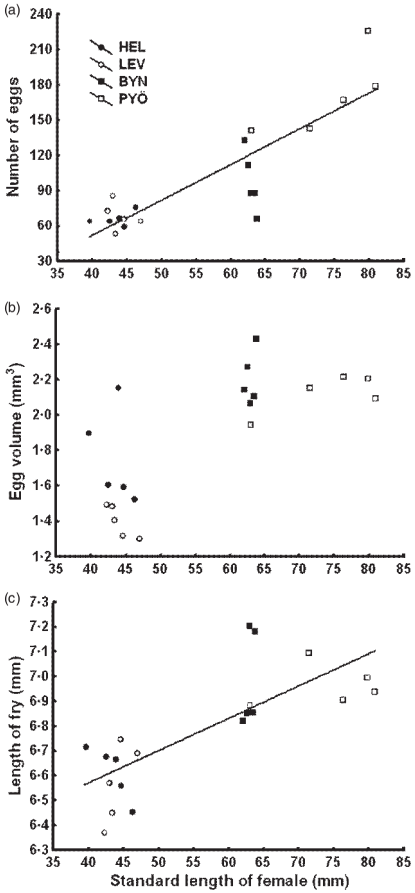


Fig. 4. Size dependence of female reproductive output (a, number of eggs; b, egg volume; c, length of fry) in *Pungitius pungitius*. HEL denotes Baltic Sea near Helsinki; LEV denotes White Sea at Levin Navolok Bay; BYN denotes Bynästjärnen (Swedish pond); and PYÖ denotes Pyöreälampi (Finnish pond). Regression lines are shown in the cases of significant relationships, only for illustrative purposes. Egg volume and length of fry are mean (per female) values.

Studies on systems with primarily female-biased SSD are rare, but the reported patterns still follow Rensch's rule in many cases (cf. Fairbairn 1997; Stephens & Wiens 2009; Stuart-Fox 2009). Inversed pattern of Rensch's rule have been found exclusively in taxa with female-biased SSD (cf. Fairbairn 1997), and the results of our intraspecific study are consistent with this pattern. This relationship in

P. pungitius appears to emerge owing to the female-biased SSD in populations characterized by extraordinary large individuals. Because (i) larger body size variation is observed among females than among males and (ii) there is no SSD in 'normal-sized' populations our results indicates that selection on female body size is driving body size differences in our system. This is expected to take effect through fecundity selection, or in other words, increased reproductive output of larger females, a phenomenon proven both within (Heins, Johnson & Baker 2003; Heins *et al*. 2005) and among *P. pungitius* populations (present study).

It is noteworthy that even though Rensch's rule is originally framed for interspecific comparisons, its predictions can be tested also at the within species level where detailed knowledge about the species' biology can help to give cues to the mechanisms behind SSD allometry (e.g. Fairbairn & Preziosi 1994; Fairbairn 2005; Teder & Tammaru 2005; Blanckenhorn *et al*. 2007). However, whereas reporting phenotypic body size/SSD trends from the wild can be interesting, such studies cannot distinguish between the alternative causes (i.e. local adaptation vs. phenotypic plasticity) behind the observed patterns. This can be extremely important considering the fact that environmental conditions can markedly influence the expression of SSD (Fairbairn 2005; Teder & Tammaru 2005). In contrast, results of our common garden experiment concurred with the pattern found in the wild. Although SSD in length was significant in all populations, SSD in body weight was only significant in the giant population. Therefore, the pattern in body weight SSD was as expected under an inverse of Rensch's rule. Even though the low number of common garden populations did not allow us to formally test for allometry in SSD, the results suggest that the presence/absence of SSD has a genetic component in *P. pungitius*.

A relevant and obvious question in this context arises: how are we to explain the differences in the degree of SSD among the genetically isolated *P. pungitius* populations? Considering that most components of selection for increased reproductive success (*i.e* fecundity selection, intrasexual competition, mate choice) favour larger body size (Wooton 1979; Clutton-Brock, Guiness & Albon 1982; Shine 1988, 1989; Andersson 1994), studies reporting selection for larger body size within species (e.g. Kingsolver & Pfennig 2004) or evolution of larger body size within lineage over geological time (e.g. Cope 1887; Alroy 1998) are not surprising. Still, a permanent increase in size is not something that characterizes the majority of natural populations, most probably because of counterbalancing evolutionary forces originating from ecological constraints and biotic interactions (e.g. Wilson 1975; Blanckenhorn 2000; Boucher *et al*. 2004). Therefore, whenever a certain population is released from these constraints, evolution towards some 'optimal' body size (certainly not a single value for diverse taxa: Meiri, Simberloff & Dayan 2005) is to be expected (Damuth 1993; Boback & Guyer 2003).

When *P. pungitius* is released from biotic environmental constraints (interspecific competition and predation) imposed by sympatric fish species (i.e. when inhabiting isolated ponds), local populations can evolve into aggressive, bold, quickly feeding giants (Herczeg *et al*. 2009a, c) facing a developmental deficit when kept in groups whereas marine (representing 'normal' populations constrained by interspecific competition and predation in nature) fish do not (Gonda *et al*. 2009a; Herczeg *et al*. 2009b). Interestingly, the size-wise 'normal' *P. pungitius* populations (standard length = 40–55 mm; e.g. Bănărescu & Paepke 2001) are not sexually dimorphic; the female-biased SSD indeed appears exclusively in populations where the mean body size is outstandingly large. The largest individuals we caught were females and exceeded 110 mm in total length (Rytilampi and Pyöreälampi populations). The observed inverse of Rensch's rule suggests that the increase in body size is driven by females as expected if driven by fecundity selection (e.g. Fairbairn 1997). Heins *et al*. (2003, 2005) have shown that clutch size is strongly depends on the mothers' body size in *P. pungitius*. In line with this, our results show that populations differing in the mean size of mothers differ also in various measures of female reproductive output. Unfortunately we lack measures of the size dependence of male reproductive success (note that in *P. pungitius* males defend territories and fight with each other), but it seems feasible to suggest that the two- to threefold increase in reproductive output per clutch giant females can achieve was the main driver of body size evolution in the pond populations of *P. pungitius*.

In summary, our results demonstrate hyper-allometry in SSD in a species where the sexes are either equally sized or females are larger than males. This translates to a pattern following an inverse of Rensch's rule. Results of the common garden experiment suggest that the presence/absence of female-biased SSD has a genetic component, and hence, representing evolutionary shifts most likely caused by selection. Significant SSD was only observed in the populations consisting of giant individuals, a fact that together with the inverse of Rensch's rule indicate that the evolution of gigantism is female-driven in *P. pungitius*. The two- to threefold increase of reproductive output per clutch in giant as compared with 'normal' females suggest that one selective agent responsible for female-driven gigantism is likely to be fecundity selection.

Acknowledgements

Victor Berger, Göran Englund, Tuomas Leinonen, Daniel Lussetti and Pirkko Siikamäki provided invaluable help in obtaining the samples. Access to the facilities of the Oulanka Research Station (University of Oulu) and the White Sea Biological Station (Russian Academy of Sciences) made the collections possible. Financial support was provided by the Academy of Finland, Ministry of Education and Centre for International Mobility (CIMO). The authors are highly indebted to Lukas Kratochvil and Wolf Blanckenhorn for their comments leading to improvements of the manuscript and John Loehr for correcting the English. The experiment was conducted under the licence of the Helsinki University Animal Experimentation Committee.

References

Abouheif, E. & Fairbairn, D.J. (1997) A comparative analysis of allometry for sexual size dimorphism: assessing Rensch's rule. *American Naturalist*, **149**, 540–562.

Alroy, J. (1998) Cope's rule and the dynamics of body mass evolution in North American fossil mammals. *Science*, **280**, 731–734.

Andersson, M. (1994) *Sexual Selection*. Princeton University Press, Princeton, New Jersey.

Bănărescu, P.M. & Paepke, H.-J. (2001) *The Freshwater Fishes of Europe. Vol. 5/III*. AULA-Verlag, Wiebelsheim.

Bell, M.A. (1984) Gigantism in threespine sticklebacks: implications for causation of body size evolution. *Copeia*, **1984**, 530–534.

Berry, J.F. & Shine, R. (1980) Sexual dimorphism and sexual selection in turtles (order Testudines). *Oecologia*, **44**, 185–191.

Blanckenhorn, W.U. (2000) The evolution of body size: what keeps organisms small? *Quarterly Review of Biology*, **75**, 385–407.

Blanckenhorn, W.U. (2005) Behavioural causes and consequences of sexual size dimorphism. *Ethology*, **111**, 977–1016.

Blanckenhorn, W.U., Dixon, A.F.G., Fairbairn, D.J., Foellmer, M.W., Gibert, P., van der Linde, K., Meier, R., Nylin, S., Pitnick, S., Schoff, C., Signorelli, M., Teder, T. & Wiklund, C. (2007) Proximate causes of Rensch's rule: does sexual size dimorphism in arthropods result from sex differences in developmental time? *American Naturalist*, **169**, 245–257.

Boback, S.M. (2003) Body size evolution in snakes: evidence from island populations. *Copeia*, **2003**, 81–94.

Boback, S.M. & Guyer, C. (2003) Empirical evidence for an optimal body size in snakes. *Evolution*, **57**, 345–351.

Boucher, S., Crete, M., Ouellet, J.P., Daigle, C. & Lesage, L. (2004) Large-scale trophic interactions: white-tailed deer growth and forest understory. *Ecoscience*, **11**, 286–295.

Case, T.J. (1978) A general explanation of insular body size trends in terrestrial vertebrates. *Ecology*, **59**, 1–18.

Case, T.J. (1979) Optimal body size and an animal's diet. *Acta Biotheoretica*, **28**, 54–69.

Clegg, S.M. & Owens, I.P.F. (2002) The 'island rule' in birds: medium body size and its ecological implications. *Proceedings of the Royal Society London B*, **269**, 1359–1365.

Clutton-Brock, T.H., Guiness, F.E. & Albon, S.D. (1982) *Red Deer: Behaviour and Ecology of Two Sexes*. University of Chicago Press, Chicago.

Colwell, R.K. (2000) Rensch's rule crosses the line: convergent allometry of sexual size dimorphism in hummingbirds and flower mites. *American Naturalist*, **156**, 495–510.

Cope, E.D. (1887) *The Origin of the Fittest*. Appleton, New York.

Cox, R.M., Kelly, S.L. & John-Adler, H.B. (2003) A comparative test of adaptive hypotheses for sexual size dimorphism in lizards. *Evolution*, **57**, 1653–1669.

Dale, J., Dunn, P.O., Figuerola, J., Lislevand, T., Székely, T. & Whittingham, L.A. (2007) Sexual selection explains Rensch's rule of allometry for sexual size dimorphism. *Proceedings of the Royal Society B*, **274**, 2971–2979.

Damuth, J. (1993) Cope's rule, the island rule and the scaling of mammalian population density. *Nature*, **365**, 748–750.

Fairbairn, D.J. (1997) Allometry for sexual size dimorphism: patterns and process in the coevolution of body size in males and females. *Annual Review of Ecology and Systematics*, **28**, 659–687.

Fairbairn, D.J. (2005) Allometry for sexual size dimorphism: testing two hypotheses for Rensch's rule in the water strider *Aquarius remigis*. *American Naturalist*, **166**(Suppl.), 69–84.

Fairbairn, D.J. & Preziosi, R.F. (1994) Sexual selection and the evolution of allometry for sexual size dimorphism in the water strider, *Aquarius remigis*. *American Naturalist*, **144**, 101–118.

Fairbairn, D.J., Blanckenhorn, W. & Székely, T. (2007) *Sex, Size and Gender Roles – Evolutionary Studies of Sexual Dimorphism*. Oxford University Press, Oxford.

Fox, C.J. (1996) Length changes in herring (*Clupea harengus*) larvae: effects of capture and storage in formaldehyde and alcohol. *Journal of Plankton Research*, **18**, 483–493.

Gittleman, J.L. (1985) Carnivore body size: ecological and taxonomic correlates. *Oecologia*, **67**, 540–554.

Gonda, A., Herczeg, G. & Merilä, J. (2009a) Habitat-dependent and-independent plastic responses to social environment in the ninespine stickleback (*Pungitius pungitius*) brain. *Proceedings of the Royal Society B*, **276**, 2085–2092.

Gonda, A., Herczeg, G. & Merilä, J. (2009b) Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? *Journal of Evolutionary Biology*, **22**, 1721–1726.

Heins, D.C., Johnson, J.M. & Baker, J.A. (2003) Reproductive ecology of the nine-spined stickleback from south-central Alaska. *Journal of Fish Biology*, **63**, 1131–1143.

Heins, D.C., Baker, J.A., De Silva, G. & Birden, E.L. (2005) Clutch characteristics of two populations of nine-spined stickleback from south-central Alaska. *Journal of Fish Biology*, **67**, 873–878.

Herczeg, G., Gonda, A. & Merilä, J. (2009a) Evolution of gigantism in nine-spined sticklebacks. *Evolution*, **63**, 3190–3200.

Herczeg, G., Gonda, A. & Merilä, J. (2009b) The social cost of shoaling covaries with predation risk in nine-spined stickleback (*Pungitius pungitius*) populations. *Animal Behaviour*, **77**, 575–580.

Herczeg, G., Gonda, A. & Merilä, J. (2009c) Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *Journal of Evolutionary Biology*, **22**, 544–552.

Johansson, F., Crowley, P.H. & Brodin, T. (2005) Sexual size dimorphism and sex ratios in dragonflies (Odonata). *Biological Journal of the Linnean Society*, **86**, 507–513.

Jones, J.W. & Hynes, H.B.N. (1950) The age and growth of *Gasterosteus aculeatus*, *Pygosteus pungitius* and *Spinachia vulgaris* as shown by their otoliths. *Journal of Animal Ecology*, **19**, 59–73.

Kingsolver, J.G. & Pfennig, D.W. (2004) Individual-level selection as a cause of Cope's rule of phyletic size increase. *Evolution*, **58**, 1608–1612.

Kratochwil, L. & Frynta, D. (2002) Body size, male combat, and the evolution of sexual dimorphism in eublepharid geckos (Squamata: Eublepharidae). *Biological Journal of the Linnean Society*, **76**, 303–314.

Kristoffersen, J.B. & Salvanes, A.G.V. (1998) Effects of formaldehyde and ethanol preservation on body and otoliths of *Maurolicus muelleri* and *Benthosema glaciale*. *Sarsia*, **83**, 95–102.

Kuusela, K. (2006) Isolated ninespine stickleback population, *Pungitius pungitius* (L.) in northeast Finland: large fish with reduced pelvis. *Verhhandlungen der Internationalen Vereinigung für Limnologie*, **29**, 1409–1412.

Lengkeek, W., Didderen, K., Cote, I.M., van der Zee, E.M., Snoek, R.C. & Reynolds, J.D. (2008) Plasticity in sexual size dimorphism and Rensch's rule in mediterranean blennies (Blenniidae). *Canadian Journal of Zoology*, **86**, 1173–1178.

Lindenfors, P., Székely, T. & Reynolds, J.D. (2003) Directional changes in sexual size dimorphism in shorebirds, gulls and alcids. *Journal of Evolutionary Biology*, **16**, 930–938.

Lislevand, T., Figuerola, J. & Székely, T. (2009) Evolution of sexual size dimorphism in grouse and allies (Aves: Phasianidae) in relation to mating competition, fecundity demands and resource division. *Journal of Evolutionary Biology*, **22**, 1895–1905.

Meiri, S., Simberloff, D. & Dayan, T. (2005) Insular carnivore biogeography: island area and mammalian optimal body size. *American Naturalist*, **165**, 505–514.

Merilä, J. (2006) Ennätyksellisen iso kymmenpiikki (*Pungitius pungitius*) Kuusamon Rytilammesta. *Luonnon Tutkija*, **110**, 91–93.

Moodie, G.E.E. (1972a) Predation, natural selection and adaptation in an unusual threespine stickleback. *Heredity*, **28**, 155–167.

Moodie, G.E.E. (1972b) Morphology, life history and ecology of an unusual stickleback (*Gasterosteus aculeatus*) in the Queen Charlotte Islands. *Canadian Journal of Zoology*, **50**, 721–738.

Moodie, G.E.E. & Reimchen, T.E. (1976) Phenetic variation and habitat differences in *Gasterosteus* populations of the Queen Charlotte Islands. *Systematic Zoology*, **25**, 49–61.

Peters, R.H. (1983) *The Ecological Implications of Body Size*. Cambridge University Press, Cambridge.

Reimchen, T.E. (1988) Inefficient predators and prey injuries in a population of giant stickleback. *Canadian Journal of Zoology*, **66**, 2036–2044.

Reimchen, T.E. (1991) Trout foraging failure and the evolution of body size in stickleback. *Copeia*, **1991**, 1098–1104.

Rensch, B. (1950) Die Abhängigkeit der relativen Sexualdifferenzvon der Körpergrösse. *Bonner Zoologische Beiträge*, **1**, 58–69.

Rensch, B. (1959) *Evolution above Species Level*. Columbia University Press, New York.

Roff, D.A. (1992) *The Evolution of Life Histories*. Chapmann & Hall, New York.

Rohlf, F.J. (2002) *tpsDig. Digitize Landmarks and Outlines, Version 1.37*. Department of Ecology and Evolution, State University of New York at Stony Brook.

Serrano-Meneses, M.A., Cordoba-Aguilar, A., Azpilicueta-Amorin, M., Gonzalez-Soriano, E. & Székely, T. (2009) Sexual selection, sexual size dimorphism and Rensch's rule in Odonata. *Journal of Evolutionary Biology*, **21**, 1259–1273.

Shikano, T., Shimada, Y., Herczeg, G. & Merilä, J. (2010) History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. *Molecular Ecology*, in press. DOI: 10.1111/j.1365-294x.2010.04553.x.

Shine, R. (1988) The evolution of large body size in females: a critique of Darwin's fecundity advantage model. *American Naturalist*, **131**, 124–131.

Shine, R. (1989) Ecological causes for the evolution of sexual size dimorphism: a review of the evidence. *Quarterly Review of Biology*, **64**, 419–461.

Simberloff, D., Dayan, T., Jones, C. & Ogura, G. (2000) Character displacement and release in the small Indian mongoose, *Herpestes javanicus*. *Ecology*, **81**, 2086–2099.

Sokal, R.R. & Rohlf, F.J. (1981) *Biometry: The Principles and Practice of Statistics in Biological Research*. W.H. Freeman, San Francisco.

Stamps, J.A. & Andrews, R.M. (1992) Estimating asymptotic size using the largest individual per sample. *Oecologia*, **92**, 503–512.

Stearns, S.C. (1992) *The Evolution of Life Histories*. Oxford Univerity Press, Oxford.'

Stephens, P.R. & Wiens, J.J. (2009) Evolution of sexual size dimorphisms in Emydid turtles: ecological dimorphism, Rensch's rule, and sympatric divergence. *Evolution*, **63**, 910–925.

Stuart-Fox, D. (2009) A test of Rensch's rule in dwarf chameleons (*Bradypodion* spp.), a group with female-biased sexual size dimorphism. *Evolutionary Ecology*, **23**, 425–433.

Székely, T., Freckleton, R.B. & Reynolds, J.D. (2004) Sexual selection explains Rensch's rule of size dimorphism in shorebirds. *Proceedings of the National Academy of Science USA*, **101**, 12224–12227.

Teder, T. & Tammaru, T. (2005) Sexual size dimorphism within species increases with body size in insects. *Oikos*, **108**, 321–334.

Wasserzug, R.J., Yang, H., Sepkoski, J.J. & Raup, D.M. (1979) The evolution of body size on islands: a computer simulation. *American Naturalist*, **114**, 287–295.

Webb, T.J. & Freckleton, R.P. (2007) Only half right: species with female-biased sexual size dimorphism consistently break Rensch's rule. *PLoS ONE* **2**: e897. Doi:10.1371/journal.pone.0000897.

Wilson, D.S. (1975) The adequacy of body size as a niche difference. *American Naturalist*, **109**, 769–784.

Wooton, R.J. (1979) Energy cost of egg production and environmental determinants of fecundity in teleost fishes. *Symposia of the Zoological Society of London*, **44**, 133–159.

Wu, Z., Li, Y. & Murray, B.R. (2006) Insular shifts in body size of rice frogs in the Zhoushan Archipelago, China. *Journal of Animal Ecology*, **75**, 1071–1080.

Ziuganov, V.V. & Zotin, A.A. (1995) Pelvic girdle polymorphism and reproductive barriers in the ninespine stickleback *Pungitius pungitius* (L.) from Northwest Russia. *Behaviour*, **132**, 1095–1105.

Received 30 October 2009; accepted 6 January 2010
Handling Editor: Shai Meiri

© 2010 The Authors. Journal compilation © 2010 British Ecological Society, *Journal of Animal Ecology*, **79**, 581–588

The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2010, **101**, 403–416. With 5 figures

Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution

GÁBOR HERCZEG*, MIRVA TURTIAINEN and JUHA MERILÄ

Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, PO Box 65, Helsinki FI-00014, Finland

Received 18 March 2010; revised 6 May 2010; accepted for publication 6 May 2010

Parallel evolution is characterised by repeated, independent occurrences of similar phenotypes in a given habitat type, in different parts of the species distribution area. We studied body shape and body armour divergence between five marine, four lake, and ten pond populations of nine-spined sticklebacks [*Pungitius pungitius* (Linnaeus, 1758)] in Fennoscandia. We hypothesized that marine and lake populations (large water bodies, diverse fish fauna) would be similar, whereas sticklebacks in isolated ponds (small water bodies, simple fish fauna) would be divergent. We found that pond fish had deeper bodies, shorter caudal peduncles, and less body armour (viz. shorter/absent pelvic spines, reduced/absent pelvic girdle, and reduced number of lateral plates) than marine fish. Lake fish were intermediate, but more similar to marine than to pond fish. Results of our common garden experiment concurred with these patterns, suggesting a genetic basis for the observed divergence. We also found large variation among populations within habitat types, indicating that environmental variables other than those related to gross habitat characteristics might also influence nine-spined stickleback morphology. Apart from suggesting parallel evolution of morphological characteristics of nine-spined sticklebacks in different habitats, the results also show a number of similarities to the evolution of three-spined stickleback (*Gasterosteus aculeatus* Linnaeus, 1758) morphology. *Biological Journal of the Linnean Society*, 2010, **101**, 403–416.

ADDITIONAL KEYWORDS: body armour, body shape, geographic variation, natural selection, predation.

INTRODUCTION

Spatial environmental heterogeneity, resulting in spatially varying selection pressures, is expected to drive phenotypic and genotypic divergence between populations of the same species (Mayr, 1963; Endler, 1977). Whereas local adaptations resulting from selection acting on heritable traits are thought to be responsible for much of the phenotypic differentiation in the wild (Schluter, 2000; Merilä & Crnokrak, 2001), random genetic drift (e.g. Lande, 1976; Lynch, 1990) and phenotypic plasticity can also influence patterns of differentiation (e.g. Miner *et al.*, 2005; Kuparinen & Merilä, 2007; Teplitsky *et al.*, 2008; Merilä, 2009). However, independent and repeated occurrence of the same phenotypes in similar habitats in the wild strongly implies the occurrence of parallel evolution driven by natural selection (e.g. Clarke, 1975; Endler, 1986; McGuigan, Chenoweth & Blows, 2005).

Model species with an extant ancestral form and a well-known phylogeographic history, including repeated, independent colonization of non-ancestral habitat types, such as the three-spined stickleback (*Gasterosteus aculeatus* Linnaeus, 1758), have advanced our understanding of evolution significantly (e.g. Bell & Foster, 1994; Shapiro *et al.*, 2004; Colosimo *et al.*, 2005). The three-spined stickleback is considered to be a species complex (Bell, 1976; Bell & Foster, 1994), with three distinct life-history forms: marine, anadromous (migrating from marine to freshwater habitats for reproduction), and freshwater

*Corresponding author. E-mail: gabor.herczeg@helsinki.fi

forms. The freshwater form can be further divided into benthic and limnetic species, which can coexist within the same lake (McPhail, 1984, 1992, 1993; Schluter & McPhail, 1992) or independently in stream and lake habitats (Berner *et al.*, 2008, 2009). Furthermore, shallow-water and open-water (Robinson, 2000), as well as lava-substrate and mud-substrate (Kristjánsson, Skúlason & Noakes, 2002), adapted forms have also been described. During the transition from the marine type to the freshwater type, repeated (parallel) evolution of several morphological features took place, including a reduction of body armour and a change in body shape: the ancestral marine/pelagic form has longer spines, more bony lateral plates, more streamlined body, and a longer caudal peduncle than its freshwater/benthic descendants (Bell & Foster, 1994).

Nine-spined sticklebacks (*Pungitius pungitius* Linnaeus, 1758) show similar diversity to three-spined sticklebacks in terms of distribution and morphological variability (e.g. Wootton, 1976, 1984; Bell & Foster, 1994). Nine-spined sticklebacks can be found in marine, lake, and river environments, but they are also able to persist in isolated ponds as the only fish species (e.g. Bănărescu & Paepke, 2001; Östlund-Nilsson, Mayer & Huntingford, 2007). *Pungitius* diverged from *Gasterosteus* ≥ 10 million years ago, but the genera share a similar phylogeographic history after the last glaciations (e.g. Shapiro, Bell & Kingsley, 2006). Hence, the nine-spined stickleback not only offers a model to study adaptive divergence and local adaptation in general, but also a model to test different questions related to parallel evolution between species (Shapiro *et al.*, 2006, 2009). Still, compared with the three-spined stickleback it is virtually unstudied. Some general information about the biology of the nine-spined stickleback is available (e.g. Jones & Hynes, 1950; Bell & Foster, 1994; Heins, Johnson & Baker, 2003; Heins *et al.*, 2005; Östlund-Nilsson *et al.*, 2007), and its morphological variation has been studied to some extent, but mainly from a taxonomical perspective (e.g. Münzig, 1969; Nelson, 1971; Keivany & Nelson, 2000, 2004), and less from an evolutionary perspective (but see McPhail, 1963; Gross, 1979; Ziuganov & Zotin, 1995).

Little attention has been paid to the evolution of nine-spined sticklebacks in isolated ponds (surface area *c.* 0.5–6 ha), where they occur as the only fish species. In these habitats sticklebacks can evolve under negligible piscine predation, and virtually in the absence of interspecific competition, but at least sometimes face intensive intraspecific competition. Recent studies have shown that an aggressive, bold, fast-growing giant phenotype has evolved repeatedly in ponds (Herczeg, Gonda & Merilä, 2009a,b; 2010), and that pond sticklebacks face an energetic constraint of group living that manifests itself as decreased growth (Herczeg, Gonda & Merilä, 2009c) and development of brain (Gonda, Herczeg & Merilä, 2009a). Furthermore, the gross brain structure of pond sticklebacks also differs from that of their marine conspecifics (Gonda, Herczeg & Merilä, 2009b). However, virtually nothing is known about the general morphology (e.g. body armour and shape) of these populations.

The overall goal of this study was to investigate body armour and body shape divergence among North European nine-spined stickleback populations from different habitats. We sampled fish from marine, lake, and pond populations to test the hypotheses that: (1) marine and lake populations would be more similar (representing the ancestral form), whereas pond populations would be divergent (descendant form) in terms of body armour and shape; and that (2) the patterns of habitat-dependent morphological variation in nine-spined sticklebacks are similar to those found in three-spined sticklebacks. Specifically, we predicted that pond sticklebacks under negligible piscine predation have reduced or lost body armour, and as an adaptation to a benthic lifestyle, have deeper bodies with shorter caudal peduncles than their marine or lake conspecifics. To ensure that the inference based on wild-caught specimens did not reflect only ontogenetic phenotypic plasticity, we also conducted a common garden experiment with two marine and two pond populations to verify the genetic basis of observed variation.

MATERIAL AND METHODS

Sampling and data acquisition

Adult fish were sampled from 19 locations, including five marine sites from three different seas (Atlantic Ocean, and Baltic and White Seas), four lakes (surface area > 20 ha), and ten ponds (surface area < 5.8 ha) between 2006 and 2009 (Fig. 1; Table 1). Sampling was carried out during the reproductive season between late May and early July with the aid of minnow traps, dipnets, and seine nets. The fish were sexed on site according to visual criteria on the basis of the males' nuptial coloration (black belly with white pelvic spines; the colour becomes even more apparent following exposure to tricaine methanesulphonate, MS222, see below), and only adult individuals were included in the analyses. As we were unable to obtain sufficient numbers of males from most marine and lake sites for statistical analyses, only non-gravid females were included in the analyses. The three sampled habitats differed from each other both in terms of size and diversity of the fish community. Marine populations are members of a diverse fish fauna, including several predatory and

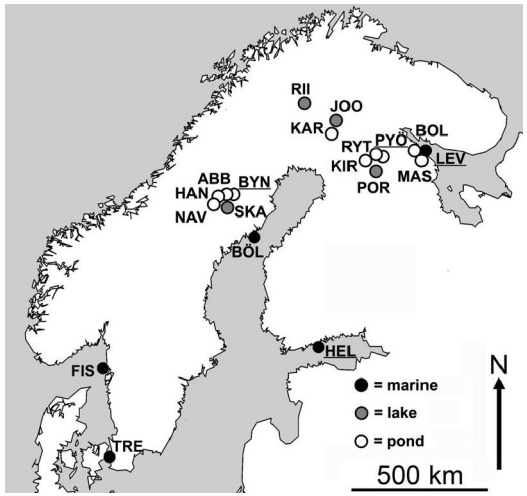


Figure 1. Map of Fennoscandia showing the locations of the nine-spined stickleback (*Pungitius pungitius*) populations studied. Underlined population codes denote populations used in the common garden experiment. For the population abbreviations, see Table 1.

numerous competing fish species. Lakes are somewhat more heterogeneous in both respects, ranging from Porontima, with a 115-ha surface area and complex fish fauna, to Joortilojärvi, with a 20-ha surface area and only recently introduced graylings, *Thymallus thymallus* (Table 1), besides the sticklebacks. Ponds are isolated (no immigration), and their fish communities are simple (Table 1). Aside from the Russian ponds Mashinnoje and Bolotnoje, where the nine-spined stickleback coexists with the three-spined stickleback (probably as a result of the relatively recent isolation from the White Sea), the nine-spined stickleback was the only fish species found in the ponds. However, different fish species have been introduced recently into some of the ponds (Table 1). It is noteworthy that apart from the relatively recently isolated Russian populations, all other pond populations are genetically isolated (Shikano *et al.*, 2010).

Collected fish were over-anaesthetized with MS222 at the site of capture, and were then stored in 96% ethanol for approximately 2 months. Following the measurement of standard length (from the tip of the mouth to the end of the tail base), fish were transferred into 4% formalin for a minimum of 2 weeks. After fixation, fish were stained with Alizarin Red S, following the methodology of Pritchard & Schluter (2001), in order to make the external bones visible. The left side of each fish was photographed with a Nikon D60 digital camera equipped with a Nikkor AF-S DX 18–55 mm f/3.5–5.6G ED lens (Nikon Corp., Tokyo, Japan) from a standard angle using a tripod. A ruler was placed in all photographs for scale. Landmarks (see below) were marked with needles.

We applied landmark-based geometric morphometrics to explore variation in body shape. Thirteen landmarks (Fig. 2) were digitized from the images using tpsDig 2 v2.10 (Rohlf, 2006). The landmarks used were as follows (Fig. 2): 1, anterior tip of upper lip; 2, posterior edge of angular; 3, anterior tip of ectocoracoid; 4, posterior tip of ectocoracoid; 5, base of first anal ray on ventral midline (VML); 6, insertion of anal fin membrane on VML; 7, origin of caudal fin membrane on VML; 8, caudal border of hypural plate at lateral midline; 9, origin of caudal fin membrane on dorsal midline (DML); 10, insertion of dorsal fin membrane on DML; 11, base of the first dorsal fin ray on DML; 12, anterior junction of the first dorsal spine on DML; 13, posterior extent of the supraoccipital. Besides the landmarks, five other morphological traits were also recorded: 1, number of dorsal spines; 2, number of anterior lateral plates (note that all fish we studied had no plates in the midsection of their bodies, so separating anterior and posterior plates

Table 1. Sampling sites, abbreviations, coordinates, surface area of water bodies, and diversity of fish communities. *N* = sample size (values in parentheses refer to sample sizes in the common garden experiment).

Sampling site	Abbr.	*N*	Coordinates	Surface area (ha)	Fish community
Marine (coastal) populations					
Fiskebackskil, Atlantic Ocean	FIS	19	58°24′N, 11°47′E	N/A	Complex
Trelleborg, Baltic Sea	TRE	23	55°38′N, 13°12′E	N/A	Complex
Bölesviken, Baltic Sea	BÖL	25	63°39′N, 20°12′E	N/A	Complex
Helsinki, Baltic Sea	HEL	26 (9)	60°13′N, 25°11′E	N/A	Complex
Levin Navolok, White Sea	LEV	29 (15)	66°18′N, 33°25′E	N/A	Complex
Lakes					
Porontima	POR	30	66°12′N, 29°16′E	115	Complex
Joortilojärvi	JOO	21	66°49′N, 26°34′E	20	*T. thymallus**
Riikojärvi	RII	22	68°06′N, 23°34′E	20	*E. lucius*†, *C. lavaretus**
Västre-Skavträsket	SKA	30	64°26′N, 19°27′E	35	Complex
Ponds					
Bolotnoje	BOL	22	66°18′N, 33°25′E	< 5 (isolated)	*G. aculeatus*
Mashinnoje	MAS	25	66°18′N, 33°25′E	< 5 (isolated)	*G. aculeatus*
Pyöreälampi	PYÖ	25 (10)	66°15′N, 29°26′E	< 5 (isolated)	*C. lavaretus**,§
Rytilampi	RYT	33	66°23′N, 29°19′E	< 5 (isolated)	–
Kirkasvetinenlampi	KIR	25	66°26′N, 29°08′E	< 5 (isolated)	*S. trutta**
Karhulampi	KAR	16	66°39′N, 26°26′E	5.8 (isolated)	*C. lavaretus*, *S. trutta**
Abbortjärn	ABB	21	64°29′N, 19°26′E	< 5 (isolated)	–
Bynästjärnen	BYN	14 (18)	64°27′N, 19°26′E	< 5 (isolated)	–
Hansmyrtjärn	HAN	24	64°33′N, 19°10′E	< 5 (isolated)	*S. trutta**
Lil-Navartjärn	NAV	25	64°33′N, 19°11′E	< 5 (isolated)	?

Coordinates of the Russian sites are approximates based on Ziuganov & Zotin (1995). *Gasterosteus aculeatus* and *Coregonus lavaretus* are larger-bodied competitors, whereas *Esox lucius, Thymallus thymallus*, and *Salmo trutta* are predators of *Pungitius pungitius*.
*Recent artificial introduction.
†Based on personal communication, unproven by our sampling.

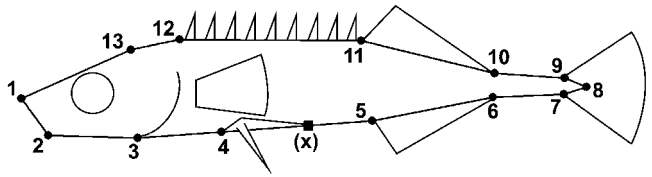


Figure 2. Illustration of the landmark positions for geometric morphometrics measurements: 1, anterior tip of upper lip; 2, posterior edge of angular; 3, anterior tip of ectocoracoid; 4, posterior tip of ectocoracoid; 5, base of first anal ray on ventral midline (VML); 6, insertion of anal fin membrane on VML; 7, origin of caudal fin membrane on VML; 8, caudal border of hypural plate at lateral midline; 9, origin of caudal fin membrane on dorsal midline (DML); 10, insertion of dorsal fin membrane on DML; 11, base of the first dorsal fin ray on DML; 12, anterior junction of the first dorsal spine on DML; 13, posterior extent of the supraoccipital; x, a landmark that was used separately from the others to measure pelvic girdle length.

was straightforward); 3, number of posterior lateral plates; 4, length of pelvic spine; 5, length of pelvic girdle. Anterior and posterior lateral plates were counted under a dissecting microscope, pelvic spine length was measured with a digital calliper, and pelvic girdle length was measured from the digital photographs with the aid of tpsDig 2, using an extra landmark at the caudal tip of the posterior process of the pelvic girdle on VML (point 'x' on Fig. 2a) in the fish that had pelvic girdles.

Common garden experiment

The common garden study was based on two marine (Baltic Sea near Helsinki and White Sea at Levin Navolok Bay) and two pond (Bynästjärnen in Sweden and Pyöreälampi in Finland) populations in order to cover habitat extremes, and to have two independent replicates per habitat type (Fig. 1). A detailed description of the common garden experiment is available in Herczeg, Gonda & Merilä (2009b). In short, five full sibling families from each population were created and ten randomly chosen individuals from every family were placed into separate 1.4-l tanks of two Allentown Zebrafish Rack Systems (Aquaneering Inc., San Diego, USA). The temperature was set to 17 °C throughout the experiment. The racks were equipped with physical, chemical, biological, and UV filters. Fish were fed two times a day in excess, first with live brine shrimp (*Artemia* sp.) nauplii, and then with frozen copepods (*Cyclops* sp.) and bloodworms (*Chironomidae*). Because of the large latitudinal differences among the source populations, we could not mimic the natural light regime. Hence, we started with a 24 h per day light period (natural during summer at high latitudes), and then gradually (over the course of one week) changed it to a 12-h light/12-h dark photoperiod after 12 weeks.

Common garden fish were over-anaesthetized with MS222 at the age of 36 weeks, when their growth had already begun to approach the asymptote (data not shown), and were photographed immediately (using the same set-up as described above). Landmarks were marked with needles, and mm graph paper was placed in each photograph for scaling. The same measurements were taken from the common garden fish as from the wild-caught ones (see above), the only difference being that we did not count the number of anterior plates here. Fish were sexed via dissection. As the data from the wild-caught fish consisted only of females, data analysed from the common garden experiment was also restricted to females. Because of other scientific projects (Gonda *et al.*, 2009a,b) and mortality, the number of females available for analyses were $N = 9$ (from four families) from the Baltic Sea population, $N = 15$ (from four families) from the White Sea, $N = 18$ (from five families) from Bynästjärnen, and $N = 10$ (from four families) from Pyöreälampi. Choice of fish for other purposes affected the families equally, whereas mortality was random. We note that the sample size in our common or garden study was low, and as a consequence, the statistical power of the analyses was also low. Hence, the results should be interpreted accordingly.

Statistical analyses

The digitized landmarks were superimposed by tpsRelw 1.46 (Rohlf, 2006). The software aligns, scales, and rotates the landmark configurations using a generalized orthogonal least-squares Procrustes procedure (Rohlf & Slice, 1990). Partial warp scores were obtained with the same program from the superimposed specimens. The next step was the relative warp analyses (on the partial warp scores), which is equal to a principal component analysis (PCA) with equal weight of all partial warps at all spatial scales. The shape visualizations (Fig. 3) were also performed by tpsRelw 1.46.

We applied two levels of analyses for the wild data. First, we ran a multivariate general linear model (GLM) on all partial warps and uniform components, with population as a fixed factor and centroid size as a covariate. The latter measure is the square root of the sum of the squared distances from the centroid to each landmark (Bookstein, 1991), and has been reported as a reliable size measure (e.g. Leinonen *et al.*, 2006). Second, we ran separate general linear mixed models (GLMMs) on the relative warps that captured more than 10% of the total variation (e.g. Berner *et al.*, 2009), with habitat as a fixed factor, population nested within habitat as a random factor, and centroid size as a covariate. Analysis of common garden data was similar to the analysis of wild data, but here the relative warps were analysed with simpler GLMMs because of the low sample size and the consequent limited statistical power: we set population as a fixed factor, family nested within population as a random factor, and centroid size as a covariate without considering interactions.

The remaining morphological variables were classified as either meristic (dorsal spine number, anterior and posterior plate numbers) or metric (pelvic girdle and pelvic spine lengths). The metric variables had to be corrected for body size. Because there were individuals or even entire populations with zero values (i.e. the pelvic spine or girdle was missing), and the populations were not homogenous with respect to size (see Herczeg *et al.*, 2009a, 2010), using residuals or entering size as a covariate to the models was not an option. Hence, we used simple ratios between pelvic spine or pelvic girdle length and standard body length (measured from the anterior tip of head to the posterior tip of the tail base; for individuals lacking a pelvic spine or girdle the ratio was zero). Although ratios have limitations (see, e.g. Albrecht, Gelvin & Hartman, 1993), in our case they represent a better choice (see above) than the use of residuals to describe relative pelvic spine or girdle length. We first ran a PCA on the five variables to get a smaller set of independent PCs. We then ran GLMMs on the PC scores with habitat as a fixed factor and population nested within habitat as a random factor. Analysis of common garden data was similar to the analysis of wild data, but here the PCs were analysed with simpler GLMMs because of the low sample size and the consequent limited statistical power: we set population as fixed factors and family nested within population as a random factor.

All analyses were performed with SPSS 15.0 (SPSS Inc. Chicago, Illinois).

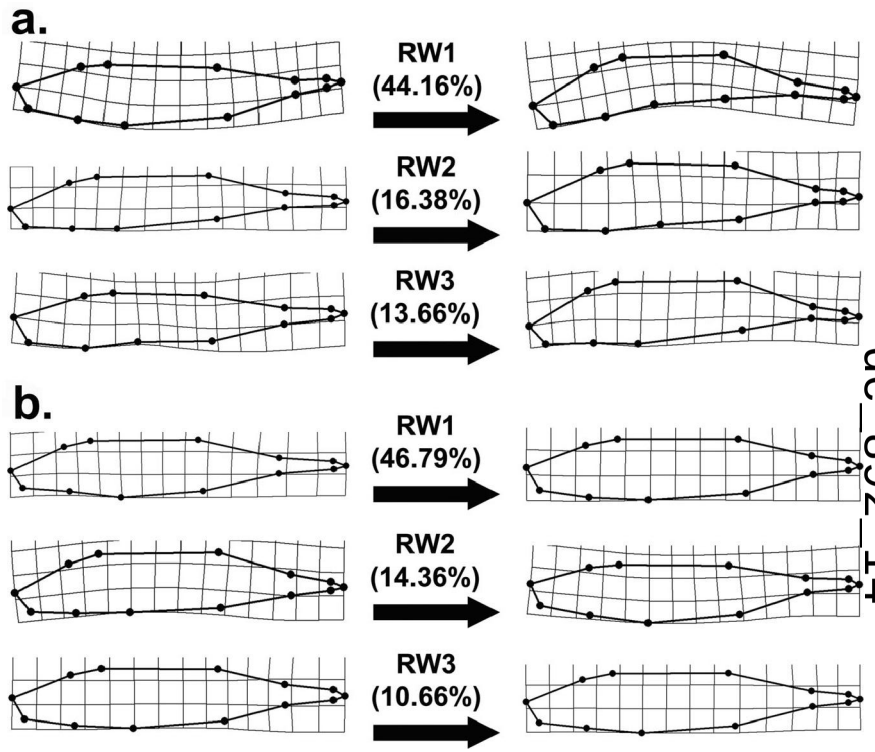


Figure 3. Results of the geometric morphometrics analyses. A, the main relative warps (RWs) of the wild data. B, the main RWs of the common garden data. The extreme phenotypes within a given RW are shown. The percentage of total variation covered by the given RWs is also shown. Note that RW1 in (a) and RW2 in (b) show bending, and are assumed to be biologically uninformative.

RESULTS

Morphological variation in the wild

The first three relative warps (RWs) described approximately 74% of the original variation in the wild data

(Fig. 3a). The first RW (44.16% of total variation) accounted for bending, which is an unwanted by-product of fixation and staining. The second and third RWs described the biologically meaningful variation, independent from bending. RW2 accounted for 16.38% of the total variance, and described a clear trend from fish with shallow bodies and long caudal peduncles towards fish with deep bodies and short caudal peduncles. RW3 (13.66% of total variance) was harder to interpret, but it appeared to describe mainly head size and mouth orientation.

The multivariate GLM revealed that the populations differed in overall shape (Wilk's $\lambda = 0.13$, $P < 0.001$) after controlling for size effects (centroid size, Wilk's $\lambda = 0.62$, $P < 0.001$; population*centroid size, Wilk's $\lambda = 0.19$, $P < 0.001$). The GLMM run on the first relative warp (describing bending) revealed no habitat effect [habitat, $F_{2,66.61} = 1.71$, $P = 0.19$; least squares (LS), mean ± SE, marine = –0.02 ± 0.01; lake = 0.01 ± 0.01; pond = 0.003 ± 0.01; centroid size, $F_{1,443.27} = 13.46$, $P < 0.001$; habitat*centroid size, $F_{2,443.47} = 0.42$, $P = 0.65$). The population effect was significant ($Z = 2.77$, $P = 0.006$). The GLMM on the second relative warp (describing a gradient from fish with shallow bodies and long caudal peduncles towards fish with deep bodies and short caudal peduncles) revealed a significant habitat effect ($F_{2,72.99} = 3.83$, $P = 0.026$; LS mean ± SE: marine = –0.006 ± 0.007; lake = –0.012 ± 0.008; pond = 0.007 ± 0.005; Fig. 4a) after correcting for size effects (centroid size, $F_{1,444.13} = 50.22$, $P < 0.001$; habitat*centroid size, $F_{2,444.26} = 2.56$, $P = 0.08$). The population effect was significant ($Z = 2.75$, $P = 0.006$). Marine and lake populations had shallow bodies with long caudal peduncles, whereas most pond populations were characterized by deep bodies and short caudal peduncles. The GLMM on the third relative warp (describing variation in head size and mouth orientation) revealed no significant habitat effect ($F_{2,118.48} = 2.55$, $P = 0.08$; LS mean ± SE, marine = 0.014 ± 0.006; lake = 0.004 ± 0.007; pond = –0.006 ± 0.004) after correcting for size effects (centroid size, $F_{1,447.00} = 15.78$, $P < 0.001$; habitat*centroid size, $F_{2,446.12} = 5.74$, $P = 0.003$). The population effect was significant ($Z = 2.71$, $P = 0.007$). Here, we found a weak trend of marine fish having somewhat smaller heads with more downward pointing mouths than lake or pond fish (data not shown).

The PCA run on the five morphological variables measured in the wild-caught fish produced two PCs with an eigenvalue of greater than 1. Based on the factor loadings (Table 2), the first PC (48.6% of total variance) described a gradient from fish with a low number of lateral plates (both anterior and posterior), together with small pelvic girdles and spines towards fish with a higher number of lateral plates and longer pelvic spines and girdles. The second PC (22.6% of total variation) revealed no significant biological information. The GLMM on PC1 revealed a significant habitat effect ($F_{2,15.97} = 10.19$, $P = 0.001$; LS mean ± SE, marine = 1.03 ± 0.30; lake = 0.05 ± 0.30; pond = –0.64 ± 0.22; Fig. 4b), and a significant population effect ($Z = 2.78$; $P = 0.005$). Marine fish typically had high numbers of lateral plates and long pelvic girdles and spines, whereas pond fish were the opposite, and lake fish were intermediate. The raw data can be found in the Appendix.

Morphological variation in the common garden fish

The first three relative warps accounted for approximately 72% of the total variation (Fig. 3b). RW1 (46.8% of the total variation) was similar to RW2 in the wild data: it described body depth and caudal peduncle length trends. RW2 (14.4% of variation) described bending, in this case originating from the minor differences in the way over-anaesthetized fish were placed for photographing. RW3 (12.1% of variation) was harder to interpret, but appeared to relate to variation in the dorsal and anal fin region.

The multivariate GLM showed that the populations differed morphologically from each other (population, Wilk's $\lambda = 0.002$, $P < 0.001$; centroid size, Wilk's $\lambda = 0.275$, $P = 0.003$). The GLMM on the first relative warp (describing a gradient from fish with shallow bodies and long caudal peduncles towards fish with deep bodies and short caudal peduncles) revealed a

Table 2. Factor loadings, eigenvalues and proportion of variance explained by the principal components (PCs) with eigenvalues >1 in the principal component analyses of nine-spined stickleback (*Pungitius pungitius*) armour traits

Wild-caught *P. pungitius*		
Trait	PC1	PC2
Number of dorsal spines	0.30	0.36
Number of anterior plates	0.69	0.57
Number of posterior plates	0.76	0.39
Relative pelvic spine length	0.84	–0.41
Relative pelvic girdle length	0.75	–0.59
Eigenvalue	2.43	1.13
Proportion of variance explained	48.57%	22.65%
Common garden *P. pungitius*		
Trait	PC1	
Number of dorsal spines	–0.47	
Number of posterior plates	0.87	
Relative pelvic spine length	0.93	
Relative pelvic girdle length	0.89	
Eigenvalue	2.64	
Proportion of variance explained	66.07%	

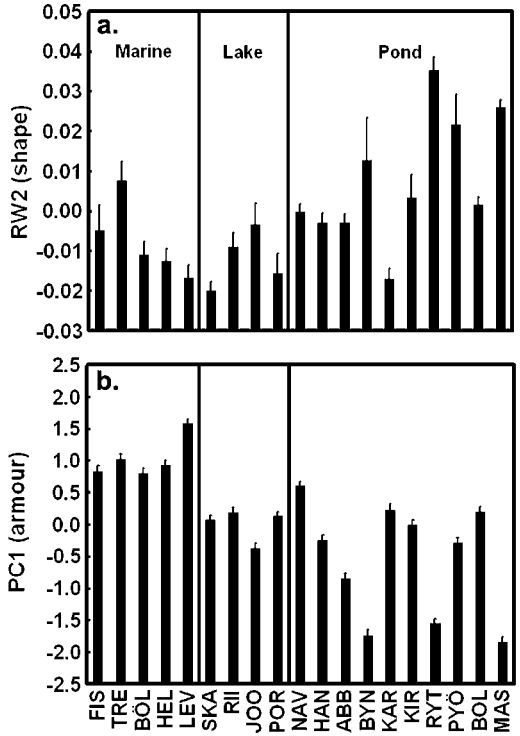


Figure 4. Significant habitat differences in nine-spined stickleback (*Pungitius pungitius*) morphology measured in wild-caught individuals. A, relative warp 2 (RW2) describes a gradient from shallow-bodied fish with long caudal peduncles (low values) towards deep-bodied fish with short caudal peduncles (high values). The RW scores are corrected for centroid size. Least-squares means (± SEs) are shown. B, principal component 1 (PC1) describes a gradient from fishes with low lateral plate numbers and short pelvic girdles and spines (low values) towards fishes with higher lateral plate numbers and longer pelvic girdles and spines (high values). Means ± (SEs) are shown. For the population abbreviations, see Table 1.

significant population effect (population, $F_{3,16.84} = 13.58$, $P < 0.001$; centroid size, $F_{1,43.26} = 2.33$, $P = 0.13$), with families within populations approaching significance ($Z = 1.85$, $P = 0.065$). Nine-spined sticklebacks from the Baltic and White Seas had shallower bodies and longer caudal peduncles than their pond conspecifics (Fig. 5a). The GLMM on the second relative warp (describing bending) revealed a significant population effect (population, $F_{3,14.52} = 3.41$, $P = 0.046$; centroid size, $F_{1,45.00} = 4.15$, $P = 0.048$), without a significant family effect ($Z = 0.78$, $P = 0.42$). For an unknown reason, Pyöreälampi fish bent differently than the other three populations (data not shown). The GLMM on the third relative warp (describing variation in the dorsal and anal fin region) revealed that the population effect was non-significant (population: $F_{3,14.90} = 2.53$, $P = 0.10$; centroid size, $F_{1,43.65} = 5.33$, $P = 0.026$), similar to the family effect ($Z = 1.64$, $P = 0.10$).

The PCA run on the four morphological variables retrieved one PC with an eigenvalueof greater than 1. Based on the factor loadings (Table 2), the first PC (66.1 % of total variation) described a gradient from fish with low posterior lateral plate number and short

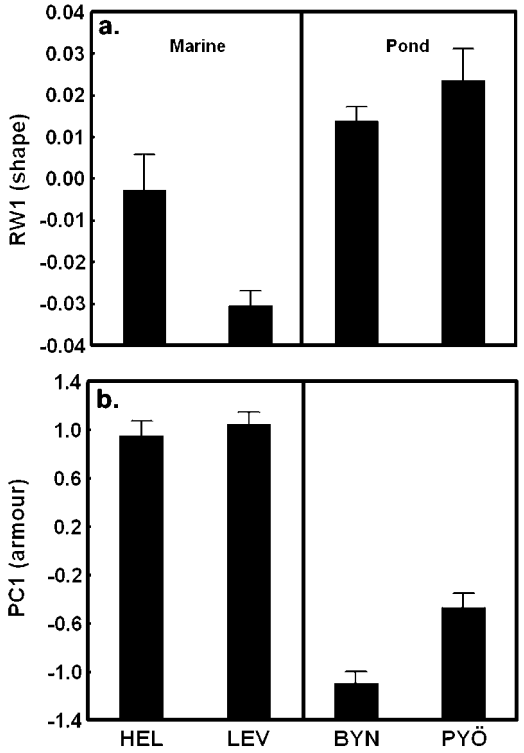


Figure 5. Significant population differences in nine-spined stickleback (*Pungitius pungitius*) morphology measured in a common garden experiment. A, relative warp 1 (RW1) describes a gradient from shallow-bodied fishes with long caudal peduncles (low values) towards deep-bodied fishes with short caudal peduncles (high values). The RW scores are corrected for centroid size. Least squares means (± SEs) are shown. B, principal component 1 (PC1) describes a gradient from fishes with low lateral plate numbers and short pelvic girdles and spines (low values) towards fishes with higher lateral plate number and longer pelvic girdles and spines (high values). Means (± SEs) are shown. For the population abbreviations, see Table 1.

pelvic spine and girdle towards fish with the opposite characteristics. The GLMM on PC1 revealed a significant population effect ($F_{3,12.80} = 105.34$, $P < 0.001$, Fig. 5b), and the family effect was non-significant ($Z = 1.10$, $P = 0.27$). Nine-spined sticklebacks from the Baltic and White Seas had a higher number of posterior lateral plates, and longer pelvic girdles and spines, than their pond conspecifics. The raw data can be found in the Appendix.

DISCUSSION

Our results provide strong support for habitat dependency in nine-spined stickleback body armour and body shape. Namely, we found that pond fish had weaker (i.e. smaller pelvic girdle and spines) or absent amour (i.e. fully absent pelvis), as well as less lateral plates, than marine fish. In addition, marine fish had shallower bodies and longer caudal peduncles than pond fish. Lake fish were intermediate in between pond and marine fish, being closer to marine fish. Patterns seen among the wild fish were supported by the patterns seen among the common garden specimens. Considering that the studied traits are heritable in sticklebacks (Hagen, 1973; Blouw & Boyd, 1992; Hermida *et al.*, 2002; Albert *et al.*, 2008; Sharpe *et al.*, 2008, Leinonen T., Cano J.M. & Merilä J., unpubl. data, that most patterns were repeatable in the laboratory, and the habitat-specific nature of the divergence, the results suggest that the observed morphological divergence reflects parallel evolution driven by habitat-specific selection pressures (e.g. Clarke, 1975; Endler, 1986; McGuigan *et al.*, 2005). We discuss these findings below in light of what is known about the functional significance of these traits, as well as compare our findings with those of earlier studies in this species and the closely related three-spined stickleback.

Body shape differentiation

Body shape evolution has been extensively studied in three-spined sticklebacks (e.g. Reimchen, Stinson & Nelson, 1985; Walker, 1997; Walker & Bell, 2000; Kristjánsson *et al.*, 2002; Kristjánsson, 2005; Hermida *et al.*, 2005; Leinonen *et al.*, 2006; Spoljaric & Reimchen, 2007; Aguirre *et al.*, 2008; Albert *et al.*, 2008; Berner *et al.*, 2008; Sharpe *et al.*, 2008; Berner *et al.*, 2009). In general, marine/pelagic sticklebacks have shallower bodies and longer caudal peduncles than their freshwater/benthic conspecifics (e.g. Bell & Foster, 1994). The functional significance of different body shapes is also resolved: a streamlined body with long and narrow caudal peduncle is optimal for prolonged steady swimming, and thus facilitates performance in pelagic environments, whereas a deep body with short caudal peduncle results in increased manoeuvrability, is optimal for short bursts of swimming, and thus facilitates performance in benthic environments (e.g. Webb, 1983; Walker, 1997; Bergstrom, 2002; Walker *et al.*, 2005).

Our data – providing the first detailed study of body shape variation in nine-spined sticklebacks – revealed habitat dependence in body depth and caudal peduncle length. Fish from marine and lake populations had shallower bodies and longer caudal peduncles than fish from pond populations, both in the wild and common garden samples. Hence, our results on body shape variation are in accordance with the expectations based on habitat-dependent optimal locomotor performance considering the differences between pelagic (marine, large lake) and benthic (pond) environments (e.g. Webb, 1983; Walker, 1997).

Reduced body armour

The function of the spines and plates of sticklebacks have been extensively studied. The main focus has been on three-spined sticklebacks, where it has been shown that the pelvic and dorsal spines form a functional unit (Reimchen, 1983) that might act as a defence against gape-limited predators (e.g. Hoogland, Morris & Tinbergen, 1957; Hagen & Gilbertson, 1972, 1973; Moodie, 1972; Gross, 1978). Lateral bony plates are also thought to serve as a defence against toothed predators, partly by forming the functional unit with the spines (Reimchen, 1983), and partly by providing a shield against injuries (Moodie, McPhail & Hagen, 1973; Reimchen, 1992, 2000). The pelvic girdle and spines are also thought to function as antipredator structures in nine-spined sticklebacks, even though their efficacy is lower than that in three-spined sticklebacks (Hoogland *et al.*, 1957). Interestingly, it has been shown that predation by aquatic insects selects against long spines both in three- (Marchinko, 2009) and nine-spined sticklebacks (Ziuganov & Zotin, 1995).

Therefore, the finding that nine-spined sticklebacks from ponds had reduced armour suggests relaxed selection for armour in freshwater habitats where predatory fishes are rare or absent. Likewise, as the density of predatory insects, which select for reduced armour in nine-spined sticklebacks (Ziuganov & Zotin, 1995), can be high in ponds, this selection pressure may have also contributed to the armour loss in pond habitats. Our results further accord with earlier studies on nine-spined sticklebacks indicating that small freshwater habitats are inhabited by fish with a reduced or absent pelvis (Nelson, 1971; Gross, 1978). Likewise, our results show that pond fish had a reduced number of lateral plates, as compared with lake and marine fish. However, lateral plates in nine-spined sticklebacks are so tiny that it is unlikely that they would have an important anti-predator function, or that they would strongly affect locomotor performance. Hence, their reduction in pond fish probably arose from either a genetic correlation with pelvic girdle/spine reduction or the higher costs of mineralizing bone in freshwater (Heuts, 1947; Giles, 1983; Bell *et al.*, 1993).

Marine and lake versus pond sticklebacks

Three-spined sticklebacks from large deep lakes resemble the ancestral marine fish in body shape, whereas fish from shallow ponds represent derived phenotypes (Walker & Bell, 2000; Spoljaric & Reimchen, 2007). Our results from nine-spined sticklebacks concur, to a large extent, with these patterns. This was expected because, aside from salinity, large lakes are more marine-like than pond-like from the

© 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2010, **101**, 403–416

perspective of sticklebacks: they are large bodies of water with complex fish communities. Furthermore, strong signs of parallel evolution were recovered in completely independent (both geographically and genetically isolated) cases: similar phenotypes occurred in Swedish, Finnish, and Russian small ponds, in the large lakes of Sweden and Finland, or in the Baltic and White Seas. As all these locations are separated by distances exceeding several hundreds of kilometres, and have been shown to be genetically isolated (Shikano *et al*., 2010), independent parallel evolution in response to similar habitat types is strongly implicated.

However, our analyses also revealed significant among-population heterogeneity in morphology within habitat types. With respect to body armour, most variation was among ponds, reflecting the differences in the levels of reduction, probably connected to the fact that predatory fish were recently introduced into some of them. Selective forces on body shape in most species, including sticklebacks, are notoriously complex, and are significantly influenced by a number of biotic and abiotic factors (e.g. Spoljaric & Reimchen, 2007). Hence, it is likely that our rough habitat characterization might have overlooked some environmental variation relevant for body shape evolution.

CONCLUSION

We found strong support for our original hypotheses: (1) pond fish were clearly divergent from marine and lake fish in terms of body shape and armour; and (2) most patterns found in the nine-spined stickleback were similar to those reported from the three-spined stickleback. Marine/lake fish had larger pelvic girdles and spines, more lateral plates, longer caudal peduncles, and more streamlined bodies than pond fish. In general, the repeated occurrence of similar morphological phenotypes in the ponds together with the earlier reported repeated evolution of giant, fast-growing, aggressive, and bold phenotypes in ponds (Herczeg *et al*., 2009a,b, 2010) is suggestive of ongoing ecological speciation as a result of adaptation to special habitats (e.g. Langerhans, Gifford & Joseph, 2007; Berner *et al*., 2009). To test this hypothesis, studies investigating possible differences in the levels and mechanisms of reproductive isolation would be needed.

ACKNOWLEDGEMENTS

We are highly indebted to Victor Berger, Jacquelin DeFaveri, Göran Englund, Lotta Kvarnemo, Tuomas Leinonen, Daniel Lussetti, Tapani Partanen, Jouko Pokela, Tuomas Perkkiö, and Pirkko Siikamäki for their help in obtaining the samples. We thank the Oulanka Research Station (University of Oulu), the White Sea Biological Station (Russian Academy of Sciences) and Kristineberg Marine Biological Station (University of Göteborg) for their help with practicalities in the field. We thank Tuomas Leinonen and two anonymous reviewers for their constructive comments leading to improvements of the manuscript, and Jacquelin DeFaveri for correcting the English. Our study was supported by the Academy of Finland (GH, JM) and by a BALTGENE-project from the BONUS+ programme. The experiment was conducted under the license of the Helsinki University Animal Experimentation Committee.

REFERENCES

Aguirre WE, Ellis KE, Kusenda M, Bell MA. 2008. Phenotypic variation and sexual dimorphism in anadromous threespine stickleback: implications for postglacial adaptive radiation. *Biological Journal of the Linnean Society* **95:** 465–478.

Albert AYK, Sawaya S, Vines TH, Knecht AK, Miller CT, Summers BR, Balabhadra S, Kingsley DM, Schluter D. 2008. The genetics of adaptive shape shift in stickleback: pleiotrophy and effect size. *Evolution* **62:** 76–85.

Albrecht GH, Gelvin BR, Hartman SE. 1993. Ratios as a size adjustment in morphometrics. *American Journal of Physical Anthropology* **91:** 441–468.

Bănărescu PM, Paepke H-J. 2001. *The freshwater fishes of Europe*, Vol. **5/III**. Wiebelsheim: AULA-Verlag.

Bell MA. 1976. Evolution of phenotypic diversity of Gasterosteus aculeatus superspecies on the Pacific Coast of North America. *Systematic Zoology* **25:** 211–227.

Bell MA, Foster SA, eds. 1994. *The evolutionary biology of the threespine stickleback*. New York: Oxford Science.

Bell MA, Orti G, Walker JA, Koenings JP. 1993. Evolution of pelvic reduction in threespine stickleback fish – a test of competing hypotheses. *Evolution* **47:** 906–914.

Bergstrom CA. 2002. Fast-start swimming performance and reduction in lateral plate number in threespine stickleback. *Canadian Journal of Zoology* **80:** 207–213.

Berner D, Adams DC, Grandchamp A-C, Hendry AP. 2008. Natural selection drives patterns of lake-stream divergence in stickleback foraging morphology. *Journal of Evolutionary Biology* **21:** 1653–1665.

Berner D, Grandchamp A-C, Hendry AP. 2009. Variable progress toward ecological speciation in parapatry: stickleback across eight lake-stream transitions. *Evolution* **63:** 1740–1753.

Blouw DM, Boyd GJ. 1992. Inheritance of reduction, loss and asymmetry of the pelvis in *Pungitius pungitius* (ninespine stickleback). *Heredity* **68:** 33–42.

Bookstein FL. 1991. *Morphometric tools for landmark data: geometry and biology*. Cambridge: Cambridge University Press.

Clarke BC. 1975. The contribution of ecological genetics to evolutionary theory: detecting the direct effects of natural selection on particularly polymorphic loci. *Genetics* **79** (Suppl.)**:** 911–922.

Colosimo PF, Hoseman KE, Balabhadra S, Villareal G Jr, Dickson M, Grimwodd J, Schmutz J, Myers RM, Schluter D, Kingsley DM. 2005. Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. *Science* **307:** 1928–1933.

Endler J. 1977. *Geographic variation, speciation and clines*. Princeton, NJ: Princeton University Press.

Endler JA. 1986. *Natural selection in the wild*. Princeton, NJ: Princeton University Press.

Giles N. 1983. The possible role of environmental calcium levels during the evolution of phenotypic diversity in Outer-Hebridean populations of the 3-spined stickleback, *Gasterosteus aculeatus*. *Journal of Zoology* **199:** 535–544.

Gonda A, Herczeg G, Merilä J. 2009a. Habitat-dependent and -independent plastic responses to social environment in the ninespine stickleback (*Pungitius pungitius*) brain. *Proceedings of the Royal Society B* **276:** 2085–2092.

Gonda A, Herczeg G, Merilä J. 2009b. Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? *Journal of Evolutionary Biology* **22:** 1721–1726.

Gross HP. 1978. Natural selection by predators on the defensive apparatus of the three-spined stickleback, *Gasterosteus aculeatus* L. *Canadian Journal of Zoology* **56:** 398–413.

Gross HP. 1979. Geographic variation in European ninespine sticklebacks, *Pungitius pungitius*. *Copeia* **1979:** 405–412.

Hagen DW. 1973. Inheritance of number of lateral plates and gill rakers on *Gasterosteus aculeatus*. *Heredity* **30:** 303–312.

Hagen DW, Gilbertson LG. 1972. Geographic variation and environmental selection in *Gasterosteus aculeatus* L. in the Pacific Northwest, America. *Evolution* **26:** 32–51.

Hagen DW, Gilbertson LG. 1973. Selective predation and the intensity of selection acting upon the lateral plates of threespine sticklebacks. *Heredity* **30:** 273–287.

Heins DC, Johnson JM, Baker JA. 2003. Reproductive ecology of the nine-spined stickleback from south-central Alaska. *Journal of Fish Biology* **63:** 1131–1143.

Heins DC, Baker JA, de Silva G, Birden EL. 2005. Clutch characteristics of two populations of nine-spined sticklebacks from south-central Alaska. *Journal of Fish Biology* **67:** 873–878.

Herczeg G, Gonda A, Merilä J. 2009a. Evolution of gigantism in nine-spined sticklebacks. *Evolution* **63:** 3190–3200.

Herczeg G, Gonda A, Merilä J. 2009b. Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *Journal of Evolutionary Biology* **22:** 544–552.

Herczeg G, Gonda A, Merilä J. 2009c. The social cost of shoaling covaries with predation risk in nine-spined stickleback (*Pungitius pungitius*) populations. *Animal Behaviour* **77:** 575–580.

Herczeg G, Gonda A, Merilä J. 2010. Rensch's rule inverted – female-driven gigantismin nine-spined stickleback (*Pungitius pungitius*). *Journal of Animal Ecology* **79:** 581–588.

Hermida M, Fernández C, Amaro R, San Miguel E. 2002. Heritability and "evolvability" of meristic characters in a natural population of *Gasterosteus aculeatus*. *Canadian Journal of Zoology* **80:** 532–541.

Hermida M, Fernández C, Amaro R, San Miguel E. 2005. Morphometric and meristic variation in Galician threespine stickleback populations, northwest Spain. *Environmental Biology of Fishes* **73:** 189–200.

Heuts MJ. 1947. Experimental studies on adaptive evolution in *Gasterosteus aculeatus* L. *Evolution* **1:** 89–102.

Hoogland R, Morris D, Tinbergen N. 1957. The spines of sticklebacks (*Gasterosteus* and *Pygosteus*) as means of defence against predators (*Perca* and *Esox*). *Behaviour* **10:** 205–236.

Jones JW, Hynes HBN. 1950. The age and growth of *Gasterosteus aculeatus, Pygosteus pungitius* and *Spinachia vulgaris* as shown by their otoliths. *Journal of Animal Ecology* **19:** 59–73.

Keivany Y, Nelson JS. 2000. Taxonomic review of the genus *Pungitius*, ninespine sticklebacks (Gasterosteidae). *Cybium* **24:** 107–122.

Keivany Y, Nelson JS. 2004. Phylogenetic relationships of sticklebacks (Gasterosteidae), with special emphasis of ninespine sticklebacks (*Pungitius* spp.). *Behaviour* **141:** 1485–1497.

Kristjánsson BK. 2005. Rapid morphological changes in threespine stickleback, *Gasterosteus aculeatus*, in freshwater. *Environmental Biology of Fishes* **74:** 357–363.

Kristjánsson BK, Skúlason S, Noakes DLG. 2002. Rapid divergence in a recently isolated population of threespine stickleback (*Gasterosteus aculeatus* L.). *Evolutionary Ecology Research* **4:** 659–672.

Kuparinen A, Merilä J. 2007. Detecting and managing fisheries-induced evolution. *Trends in Ecology and Evolution* **22:** 652–659.

Lande R. 1976. Natural selection and random genetic drift in phenotypic evolution. *Evolution* **30:** 314–334.

Langerhans RB, Gifford ME, Joseph EO. 2007. Ecological speciation in *Gambusia* fishes. *Evolution* **61:** 2056–2074.

Leinonen T, Cano JM, Mäkinen H, Merilä J. 2006. Contrasting patterns of body shape and neutral genetic divergence in marine and lake populations of threespine sticklebacks. *Journal of Evolutionary Biology* **19:** 1803–1812.

Lynch M. 1990. The rate of morphological evolution in mammals from the standpoint of the neutral expectation. *American Naturalist* **136:** 727–741.

McGuigan K, Chenoweth SF, Blows MW. 2005. Phenotypic divergence along lines of genetic variance. *American Naturalist* **165:** 32–43.

McPhail JD. 1963. Geographic variation in North American ninespine sticklebacks, *Pungitius pungitius*. *Journal of the Fisheries Research Board of Canada* **20:** 27–44.

McPhail JD. 1984. Ecology and evolution sympatric sticklebacks (*Gasterosteus*): morphological and genetic evidence for a species pair in Paxton Lake, Texada Island, British Columbia. *Canadian Journal of Zoology* **62:** 1402–1408.

McPhail JD. 1992. Ecology and evolution sympatric sticklebacks (*Gasterosteus*): evidence for a species pair in Paxton Lake, Texada Island, British Columbia. *Canadian Journal of Zoology* **70:** 361–369.

McPhail JD. 1993. Ecology and evolution sympatric stickle-

backs (*Gasterosteus*): origin of the species pairs. *Canadian Journal of Zoology* **71:** 515–523.

Marchinko KB. 2009. Predation's role in repeated phenotypic and genetic divergence of armor in threespine stickleback. *Evolution* **63:** 127–138.

Mayr E. 1963. *Animal species and evolution*. Cambridge: Harvard University Press.

Merilä J. 2009. It's the genotype, stupid! *Journal of Animal Breeding and Genetics* **126:** 1–2.

Merilä J, Crnokrak P. 2001. Comparison of genetic differentiation at marker loci and quantitative traits. *Journal of Evolutionary Biology* **14:** 892–903.

Miner BG, Sultan SE, Morgan SG, Padilla DK, Relyea RA. 2005. Ecological consequences of phenotypic plasticity. *Trends in Ecology and Evolution* **20:** 685–692.

Moodie GEE. 1972. Predation, natural selection and adaptation in an unusual threespine stickleback. *Heredity* **28:** 155–167.

Moodie GEE, McPhail JD, Hagen DW. 1973. Experimental demonstration of selective predation on *Gasterosteus aculeatus*. *Behaviour* **47:** 95–105.

Münzig J. 1969. Variabilität, Verbreitung und Systematik der Arten und Unterarten in der Gattung *Pungitius* Coste, 1848. (Pisces, Gasterosteidae). *Sonder Zeitschrift für zoologische Systematik und Evolutionsforschung* **7:** 208–233.

Nelson JS. 1971. Absence of the pelvic complex in ninespine sticklebacks, *Pungitius pungitius*, collected in Ireland and Wood Buffalo National Park Region, Canada, with notes on mersitic variation. *Copeia* **1971:** 707–717.

Östlund-Nilsson S, Mayer I, Huntingford FA. 2007. *Biology of the three-spined stickleback*. Boca Raton: CRC Press.

Pritchard JR, Schluter D. 2001. Declining interspecific competition during character displacement: summoning the ghost of competition past. *Evolutionary Ecology Research* **3:** 209–220.

Reimchen TE. 1983. Structural relationships between spines and lateral plates in threespine stickleback (*Gasterosteus aculeatus*). *Evolution* **37:** 931–946.

Reimchen TE. 1992. Injuries on stickleback from attacks by a toothed predator (*Oncorhynchus*) and implications for the evolution of lateral plates. *Evolution* **46:** 1224–1230.

Reimchen TE. 2000. Predator handling failures of lateral plate morphs in *Gasterosteus aculeatus*: functional implications for the ancestral plate condition. *Behaviour* **137:** 1081–1096.

Reimchen TE, Stinson EM, Nelson JS. 1985. Multivariate differentiation of parapatric and allopatric populations of threespine stickleback in the Sangan River watershed, Queen Charlotte Islands. *Canadian Journal of Zoology* **63:** 2944–2951.

Robinson BW. 2000. Trade offs in habitat-specific foraging efficiency and the nascent adaptive divergence of sticklebacks in lakes. *Behaviour* **137:** 865–888.

Rohlf FJ. 2006. *TPS software series*. Distributed by the author. Stony Brook, NY: Department of Ecology and Evolution, State University of New York.

Rohlf FJ, Slice DS. 1990. Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Zoology* **39:** 40–59.

Schluter D. 2000. *The ecology of adaptive radiation*. Oxford: Oxford Univerity Press.

Schluter D, McPhail JD. 1992. Ecological character displacement and speciation in sticklebacks. *American Naturalist* **140:** 85–108.

Shapiro MD, Marks ME, Peichel CL, Blackman BK, Nereng KS, Jónsson B, Schluter D, Kingsley DM. 2004. Genetic and developmental basis of evolutionary pelvis reduction in threespine sticklebacks. *Nature* **428:** 717–723.

Shapiro MD, Bell MA, Kingsley DM. 2006. Parallel genetic origins of pelvic reductions of vertebrates. *Proceedings of the National Academy of Sciences USA* **103:** 13753–13758.

Shapiro MD, Summers BR, Balabhadra S, Aldenhoven JT, Miller AL, Cunningham CB, Bell MA, Kingsley DM. 2009. The genetic architecture of skeletal convergence and sex determination of ninespine sticklebacks. *Current Biology* **19:** 1140–1145.

Sharpe DMT, Räsänen K, Berner D, Hendry AP. 2008. Genetic and environmental contributions to the morphology of lake and stream stickleback: implications for gene flow and reproductive isolation. *Evolutionary Ecology Research* **10:** 849–866.

Shikano T, Shimada Y, Herczeg G, Merilä J. 2010. History *vs.* habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. *Molecular Ecology* **19:** 1147–1161.

Spoljaric MA, Reimchen TE. 2007. 10000 years later: evolution of body shape in Haida Gwaii three-spined stickleback. *Journal of Fish Biology* **70:** 1484–1503.

Teplitsky C, Mills JA, Alho JS, Yarall JW, Merilä J. 2008. Bergmann's rule and climate change: disentangling environmental and genetic responses in a wild bird population. *Proceedings of the National Academy of Sciences USA* **105:** 13492–13496.

Walker JA. 1997. Ecological morphology of lacustrine threespine stickleback *Gasterosteus aculeatus* L. (Gasterosteidae) body shape. *Biological Journal of the Linnean Society* **61:** 3–50.

Walker JA, Bell MA. 2000. Net evolutionary trajectories of body shape evolution within a microgeographic radiation of threespine sticklebacks (*Gasterosteus aculeatus*). *Journal of Zoology* **252:** 293–302.

Walker JA, Ghalambor CK, Griset OL, McKenney D, Reznick DN. 2005. Do faster starts increase the probability of evading predators? *Functional Ecology* **19:** 808–815.

Webb PW. 1983. Speed, acceleration and manoeuvrability of two teleost fishes. *Journal of Experimental Biology* **102:** 115–122.

Wootton RJ. 1976. *The biology of sticklebacks*. London: Academic Press.

Wootton RJ. 1984. *The functional biology of sticklebacks*. Berkeley, CA: University of California Press.

Ziuganov VV, Zotin AA. 1995. Pelvic girdle polymorphism and reproductive barriers in the ninespine stickleback *Pungitius pungitius* (L.) from northwest Russia. *Behaviour* **132:** 1095–1105.

APPENDIX

Mean (±SD) values of the morphological traits in different *Pungitius pungitus* populations. The minimum–maximum range is given in parentheses. The underlined population codes denote common garden data; *N*, sample size. For the population abbreviations, see Table 1 and Figure 1.

Population	*N*	Dorsal spine no.	Anterior plate no.	Posterior plate no.	Relative pelvic girdle length	Relative pelvic spine length	Standard length (mm)
FIS	19	8.89 ± 0.46 (8–10)	3.05 ± 1.18 (0–5)	9.52 ± 2.41 (5–15)	0.184 ± 0.011 (0.170–0.206)	0.105 ± 0.012 (0.074–0.120)	38.99 ± 2.29 (35.88–42.49)
TRE	23	9.70 ± 0.70 (9–11)	3.26 ± 1.29 (0–5)	10.61 ± 1.70 (7–13)	0.157 ± 0.010 (0.141–0.182)	0.117 ± 0.013 (0.084–0.147)	33.67 ± 5.34 (28.87–56.27)
BÖL	25	9.76 ± 0.52 (9–11)	2.92 ± 1.19 (0–5)	9.40 ± 1.63 (6–13)	0.165 ± 0.009 (0.147–0.180)	0.109 ± 0.012 (0.088–0.130)	42.71 ± 3.82 (36.27–50.26
HEL	26	9.96 ± 0.44 (9–11)	2.96 ± 1.11 (1–5)	9.35 ± 1.96 (6–13)	0.172 ± 0.008 (0.157–0.191)	0.115 ± 0.012 (0.088–0.142)	38.41 ± 5.01 (31.21–49.46)
HEL	9	9.55 ± 0.53 (9–10)	–	9.44 ± 1.81 (7–13)	0.151 ± 0.013 (0.125–0.172)	0.096 ± 0.008 (0.082–0.107)	50.77 ± 2.41 (45.03–53.71)
LEV	29	9.65 ± 0.48 (9–10)	4.03 ± 0.94 (2–6)	14.0 ± 2.08 (10–18)	0.170 ± 0.011 (0.149–0.186)	0.107 ± 0.010 (0.089–0.127)	39.74 ± 3.82 (33.79–49.99)
LEV	15	9.53 ± 0.52 (9–10)	–	13.00 ± 1.60 (10–15)	0.138–0.005 (0.130–0.147)	0.090 ± 0.004 (0.078–0.095)	62.08 ± 3.46 (57.55–69.59)
POR	30	9.97 ± 0.32 (9–11)	2.37 ± 0.89 (0–4)	7.33 ± 0.71 (6–9)	0.143 ± 0.006 (0.128–0.156)	0.088 ± 0.009 (0.065–0.101)	39.93 ± 3.26 (33.63–50.30)
JOO	21	9.24 ± 0.44 (9–10)	0.57 ± 0.81 (0–3)	6.19 ± 0.93 (4–8)	0.151 ± 0.112 (0.127–0.176	0.094 ± 0.010 (0.075–0.110)	37.57 ± 4.09 (31.55–46.95)
RII	22	9.36 ± 0.58 (8–10)	1.68 ± 0.89 (0–3)	8.04 ± 1.25 (6–11)	0.171 ± 0.010 (0.150–0.188)	0.088 ± 0.005 (0.078–0.099)	53.43 ± 4.90 (43.78–62.38)
SKA	30	9.53 ± 0.57 (9–11)	1.97 ± 0.72 (1–4)	9.23 ± 1.25 (7–13)	0.132 ± 0.006 (0.121–0.146)	0.081 ± 0.008 (0.063–0.096)	45.05 ± 6.53 (36.53–60.36)
BOL	22	9.64 ± 0.66 (9–11)	3.23 ± 0.81 (2–5)	10.64 ± 2.38 (6–14)	0.125 ± 0.046 (0.019–0.166)	0.051 ± 0.037 (0–0.103)	45.77 ± 4.14 (39.70–57.34)
MAS	25	9.60 ± 0.50 (9–10)	1.84 ± 1.03 (0–4)	7.20 ± 0.96 (6–10)	0	0	51.56 ± 5.72 (38.44–60.20)
PYÖ	25	10.08 ± 0.40 (9–11)	1.68 ± 0.69 (0–3)	6.00 ± 0.71 (4–7)	0.144 ± 0.008 (0.123–0.156)	0.072 ± 0.007 (0.058–0.089)	72.23 ± 5.26 (58.14–78.99)
PYÖ	10	10.00 ± 0 (10–10)	–	5.90 ± 0.88 (5–7)	0.120 ± 0.005 (0.109–0.127)	0.063 ± 0.002 (0.060–0.066)	78.86 ± 2.31 (74.62–80.95)
RYT	33	9.54 ± 0.67 (8–11)	1.45 ± 0.75 (0–3)	5.24 ± 0.71 (4–7)	0.087 ± 0.017 (0.032–0.112)	0	64.35 ± 7.78 (51.94–80.51)
KIR	25	9.32 ± 0.48 (9–10)	2.32 ± 0.69 (1–3)	7.64 ± 0.95 (6–10)	0.151 ± 0.007 (0.134–0.167)	0.073 ± 0.016 (0–0.086)	61.20 ± 4.06 (54.39–72.16)
KAR	16	9.50 ± 0.52 (9–10)	0.69 ± 0.95 (0–3)	6.87 ± 1.02 (5–9)	0.186 ± 0.014 (0.158–0.221)	0.118 ± 0.008 (0.104–0.133)	43.59 ± 3.96 (38.30–51.65)
ABB	21	9.38 ± 0.74 (8–11)	0.48 ± 0.68 (0–2)	5.81 ± 0.98 (4–8)	0.137 ± 0.010 (0.121–0.159)	0.055 ± 0.010 (0.040–0.078)	51.81 ± 5.07 (44.31–64.42)
BYN	14	6.43 ± 2.79 (0–10)	0.29 ± 0.47 (0–1)	7.00 ± 1.88 (5–12)	0.120 ± 0.014 (0.094–0.140)	0	61.80 ± 2.76 (57.04–65.95)
BYN	18	9.78 ± 0.43 (9–10)	–	5.55 ± 0.70 (4–7)	0.100 ± 0.010 (0.078–0.114)	0.035 ± 0.018 (0–0.055)	69.38 ± 3.00 (64.90–76.32)
HAN	24	9.96 ± 0.69 (9–11)	1.17 ± 1.34 (0–4)	7.75 ± 1.51 (5–12)	0.138 ± 0.009 (0.119–0.156)	0.073 ± 0.018 (0–0.095)	45.11 ± 4.86 (35.93–56.28)
NAV	25	9.84 ± 0.62 (8–11)	2.48 ± 0.77 (1–4)	11.32 ± 1.70 (8–16)	0.148 ± 0.009 (0.129–0.161)	0.081 ± 0.007 (0.069–0.092)	49.27 ± 3.71 (35.99–54.45)

JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12103

Evolution of stickleback feeding behaviour: genetics of population divergence at different ontogenetic stages

G. HERCZEG*†, N. I. AB GHANI* & J. MERILÄ*

*Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, Helsinki, Finland
†Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Budapest, Hungary

Keywords:

animal personality;
behavioural consistency;
ontogeny;
predation;
nine-spined stickleback.

Abstract

The evolutionary significance of individual consistency in a given behaviour – called animal personality – has been subject to a lot of recent research. However, the genetic underpinnings of population divergence in mean personality have rarely been studied, especially across different ontogenetic stages. Previous work has shown that marine vs. pond populations of nine-spined sticklebacks (*Pungitius pungitius*) have undergone adaptive divergence in a series of fitness-related traits, including behaviour. One particular behavioural trait important in this system is feeding activity: giant pond sticklebacks are more active feeders than their normal sized marine conspecifics. In a common garden experiment, we raised individuals from pure and hybrid F_1-generation crosses of a highly divergent marine – pond population pair to see if (i) feeding activity and/or its ontogenetic change was consistent between individuals, and if (ii) population divergence at different ontogenetic stages could be explained by additive genetic, nonadditive genetic or maternal effects. We found that feeding activity decreased with age, but that these changes were consistently different among both individuals and crosses. The among cross patterns were consistent with a nonadditive genetic scenario: in the early period pond sticklebacks expressed dominance for high feeding activity, while in the late period marine sticklebacks expressed dominance for low feeding activity. We conclude that nine-spined sticklebacks exhibit different feeding personalities, and that the population divergence in feeding personality is explainable by age-dependent expression of genetic dominance.

Introduction

The importance of individual consistency in behaviour, resulting in consistent behavioural differences between individuals in similar contexts and making the individual behavioural repertoire narrower than what is seen in the whole population, has become widely recognized recently. The phenomenon, that is, individual consistency in a certain behaviour, bears close resemblance to human personality and is thus often called animal personality (e.g. Gosling, 2001). Animal personality is not to be confused with behavioural syndromes (Sih *et al.*, 2004a,b; Bell, 2007; Garamszegi *et al.*, 2012) or temperament (e.g. Réale *et al.*, 2007). Behavioural syndromes are correlations between different behaviours, while temperament is a hypothetical individual attribute expressed in different contexts as different behaviours and thus offers a possible explanation for both animal personality and behavioural syndromes. Examples of animal personality are accumulating in different behavioural traits and in a wide variety of taxa (see e.g. Table 1 in Smith & Blumstein, 2008). Further, the heritability of animal personality (i.e. consistent individual behaviour) has been estimated in different systems (Dingemanse *et al.*, 2002, 2009; Drent *et al.*, 2003; van Oers *et al.*, 2004) and its fitness consequences have also been demonstrated even at a meta-analytical level (Smith & Blumstein, 2008). If animal personality is heritable and has fitness consequences, local adaptations in populations experiencing different environments are expected. However, studies estimating the quantitative genetic parameters of population divergence in mean personality are scarce (but see Dingemanse *et al.*, 2012), and especially, estimating these parameters across different ontogenetic stages (i.e. comparing ontogenetic personality shifts) are lacking.

In a recent study, Dingemanse *et al.* (2010) described a method (individual behavioural reaction norm approach) where by measuring behaviour along an environmental gradient, individual consistency in behaviour (animal personality), the effect of the environmental gradient (behavioural plasticity) and the interaction between the two (individual consistency in behavioural plasticity) can all be estimated. This framework can be used to obtain an unbiased estimate of personality, but also, if individual behaviour is measured at different ontogenetic stages instead of an environmental gradient, personality itself and the individual differences in ontogenetic personality change can be both assessed. This might be especially useful given that ontogenetic change in behaviour, as well as individual differences in that change, are intuitively to be expected. Furthermore, populations might not only differ in their mean personality, but also in their mean ontogenetic behavioural reaction norms. In theory, behaviour (or personality) can be under different selective forces and be regulated by different genetic mechanisms at different ontogenetic stages. By utilizing specific crossing designs between populations (e.g. Lynch & Walsh, 1998), the quantitative genetic basis [viz. additive genetic, nonadditive (e.g. dominance) genetic, and maternal effects] of divergent traits can be assessed, and with proper sampling design, the assessment can be done across different ontogenetic stages.

The nine-spined stickleback (*Pungitius pungitius*) provides an excellent model to study the genetic underpinnings of population divergence in personality and in the possible ontogenetic personality change, because it has a wide distribution and can be found in a number of ecologically divergent habitats (Bănărescu & Paepke, 2001; Östlund-Nilsson *et al.*, 2007). Previous work has revealed that marine (high and diverse piscine predation) and isolated pond populations (zero piscine predation) differ in a series of behavioural, morphological and life-history traits. In particular, pond populations can reach giant sizes compared with marine sticklebacks, have reduced body armour, grow for longer periods, have higher reproductive output, mature later and have higher feeding activity probably as adaptations to reduced predation risk and interspecific competition coupled with increased intraspecific competition (Herczeg *et al.*, 2009a, b, 2010a,b, 2012; Herczeg & Välimäki, 2011; Shimada *et al.*, 2011; Ab Ghani *et al.*, 2012, 2013; Välimäki & Herczeg, 2012). Therefore, ontogenetic shifts in feeding activity and population divergence in that shift can both be expected.

In this study, we addressed the following questions. Does the nine-spined stickleback exhibit 'feeding personalities' (i.e. individual consistency in feeding activity)? Is there an ontogenetic shift in feeding activity, and if so, are there consistent differences among individuals in that shift? We know from earlier work that nine-spined stickleback populations differ in mean feeding activity (Herczeg *et al.*, 2009b; Herczeg & Välimäki, 2011), but whether there are also population differences in patterns of feeding activity across ontogeny is as yet not known. Finally, we explored the question as to what degree the population divergence detected at any ontogenetic stage can be explained by additive, and/or nonadditive genetic and/or maternal effect influences. To this end, we utilized a common garden experiment with a simple quantitative genetic design: we reared (i) pure crosses of marine sticklebacks, (ii) pure crosses of pond sticklebacks and hybrid crosses with (iii) marine fathers and pond mothers vs. (iv) pond fathers and marine mothers. For all individuals from all cross-types, we recorded feeding activity monthly during the 9 months following hatching. The expectations based on a breeding design like this are simple (Wright 1978; for applications see, for example, Laugen *et al.*, 2002; Ab Ghani *et al.*, 2012): if the population divergence is governed mainly by additive genetic effects, the pure crosses should clearly differ, and the hybrids should be similar to each other and express an intermediate phenotype to the pure crosses. If nonadditive genetic and/or maternal effects are also important, the hybrids should differ from each other and/or deviate from intermediacy. For instance, under simple dominance, the hybrids should be similar and resemble one of the pure crosses, while under maternal effects the hybrids should differ and resemble the pure cross of their maternal origin. Hence, by adopting this design, we were able to address all the above listed questions.

Correspondence: Gábor Herczeg, Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, PO Box 65, Helsinki FI-00014, Finland.
Tel.: +358 9 191 57707; fax: +358 9 191 57694;
e-mail: gaborherczeg@caesar.elte.hu

Table 1 Results from the repeated measures General Linear Mixed Model of the feeding activity of nine-spined sticklebacks. Fixed effects are shown here, random effects are reported in the text.

Effect	ndf	ddf	*F*	*P*
Origin of father (F)	1	82.8	8.53	0.0045
Origin of mother (M)	1	82.8	46.53	< 0.0001
Month of measurement (Mo)	8	402	64.86	< 0.0001
F × M	1	82.8	5.23	0.025
F × Mo	8	402	1.81	0.073
M × Mo	8	402	3.29	0.0012
F × M × Mo	8	402	5.07	< 0.0001

ndf, denotes the numerator.
ddf, the denominator degrees of freedom.

Materials and methods

The experiment

Adult nine-spined sticklebacks were collected from two populations in the late spring – early summer of 2010 at the onset of the breeding season. Fish were captured in minnow traps or with seine net. The marine population was sampled from the Baltic Sea near Helsinki, Finland (60°12′09″ N, 25°10′58″ E), while the pond population was Pyöreälampi from the Kuusamo area, Finland (66°15′40″ N, 29°26′00″ E). The populations are both geographically (approximately 900 km) and genetically (Shikano *et al.*, 2010) isolated. The marine site is characterized by very low salinity (< 6 psu; Shimada *et al.*, 2011), and the isolated pond has < 5 ha surface area. Marine sticklebacks are members of a complex fish community including several piscine predators, while in Pyöreälampi – apart from a few recently introduced small whitefish (*Coregonus lavaretus*), which are function more as competitors than predators of sticklebacks based on their diet (Kahilainen *et al.*, 2004) – the nine-spined stickleback is the only fish species. Cannibalism and aquatic insect predation at the fry stage might be present in both populations. The two sampled populations are known to exhibit large divergence in size, growth strategy, female reproductive output and feeding activity (Herczeg *et al.*, 2009a,b, 2010a, 2012; Herczeg & Välimäki, 2011; Shimada *et al.*, 2011). The drastic difference in predatory regime has resulted in increased lifespan and reduced body armour in Pyöreälampi fish (Herczeg *et al.*, 2009a, 2010b).

The experiment is the same as the one described in Ab Ghani *et al.* (2012). Briefly, we produced four types of crosses (10 full-sib families in each) via *in vitro* fertilizations: pure marine (HEL-HEL), pure pond (PYÖ-PYÖ) and hybrids with marine father and pond mother (HEL-PYÖ) or pond father and marine mother (PYÖ-HEL). All crosses were done soon after capture, so no systematic maternal 'conditioning' was applied. After hatching, five individuals from every family (five individuals × 10 families × four cross-types = 200 individuals) were placed in 1.4 L containers of four Allentown Zebrafish Rack Systems (Aquaneering Inc., San Diego, CA, USA) fitted with physical, chemical, biological and UV filters. Photoperiod was set to a 14 h light: 10 h dark periodism. Water temperature was 17 °C and all rearing was done in freshwater. Feeding was started with live brine shrimp (*Artemia* sp.) nauplii and was gradually changed to frozen bloodworms (Chironomid larvae) after 2 months. Feeding was done twice a day in excess. The tanks were separated by white plastic panels, so the fish could not see each other.

Feeding activity was quantified as the time needed for the first bite during a normal morning feeding event (Herczeg *et al.*, 2009b; Herczeg & Välimäki, 2011). Each container had a feeding hole at the top positioned at the front side of the tank. Food was provided with a pipette through the hole and the elapsed time till the first biting attempt was measured with a stopwatch. If a fish did not initiate feeding after 3 min, we terminated the observation and the individuals were assigned a time of 180s. Feeding activity was first measured 30 days after hatching, and the measurement was repeated monthly afterwards resulting in nine measurements for every individual. Every fish was measured at the exact same age with 1 day precision. During this period, six individuals died, so our data set consists of 194 sticklebacks.

Statistical analyses

Feeding activity was $\log_{10}$ transformed. To test for individual consistency in feeding personality, an ontogenetic behavioural shift, individual consistency in the expected ontogenetic behavioural shift and the importance of father and mother population of origin, a repeated measures General Linear Mixed Model (GLMM) as implemented in PROC MIXED in SAS (Littell *et al.*, 2006) was performed. Feeding activity was fitted as the response variable, origin of father and origin of mother (marine vs. pond) as fixed factors, time of measurement (month) as a repeated measures factor and individual and family (the latter nested in origin of father × origin of mother) as random factors. Interactions up to three-way between the fixed and repeated measures factors were considered fixed effects and the individual × month interaction as a random effect. We tried several covariance structures (unstructured, auto regressive, toeplitz, heterogenous toeplitz and heterogenous first order autoregressive) for the repeated measures factor, and based on Akaike Information Criterion (Littell *et al.*, 2000), we chose the model with heterogenous first order autoregressive covariance structure.

For a more detailed analysis of the quantitative genetic basis of feeding activity at different ontogenetic stages, we first ran a principal component analysis (PCA) on the nine feeding activity measures to gather a reduced set of independent variables describing different aspects of the ontogeny of behaviour. Then, using the independent PCs, we ran GLMMs with origin of father, origin of mother and their interaction as fixed effects and family nested in origin of father × origin of mother as random effect. Here, we applied Tukey–Kramer pairwise post hoc tests to directly compare the different cross-types.

We note that our particular breeding design was not optimal (cf. low sample size, low number of families, full-sib families) to assess within-population genetic variation to a high degree of accuracy. Hence, the family effect was only included in the GLMMs to account for the nonindependence of individuals, and thus the family effects should be considered with caution.

Statistical analyses were carried out using the SAS 9.2 (SAS institute Inc., Cary, NC, USA) and PASW Statistics 18 (PASW Inc., Chicago, IL, USA) softwares.

Results

General Linear Mixed Model revealed a significant individual × month interaction on feeding activity (individual: $Z = 1.48$, $P = 0.14$; individual × month: $Z = 8.18$; $P < 0.0001$). When the interaction was removed, the term 'individual' became highly significant ($Z = 3.24$, $P = 0.0012$). Hence, individuals differed consistently and significantly in their feeding activity, supporting the presence of a 'feeding personality'. More importantly, the ontogenetic shift (a decrease in feeding activity, see Fig. 1) was also individual-dependent, supporting the presence of different ontogenetic feeding personalities among individual sticklebacks. The family effect was not significant ($Z = 0.51$, $P = 0.61$).

The GLMM also revealed a significant three-way interaction between origin of father, origin of mother and month (Table 1). This result indicates that the mean ontogenetic shift in feeding activity differed between the different crosses, and the divergence cannot be explained by additive genetic effects only. A visual inspection of the data shows that the divergence between the pure crosses is clear, that is, PYÖ-PYÖ fish are more ready to eat at any ontogenetic stage than HEL-HEL fish (Fig. 1). However, while the hybrids were somewhat in between the pure lines, the pattern was not straightforward to interpret; seemingly, hybrids behaved more like pure pond fish in the early stages of ontogeny, and more like pure marine fish later on (Fig. 1).

To further clarify the ontogenetic patterns of feeding activity, a PCA on the feeding activity measures was conducted. Two biologically meaningful PCs (eigenvalues > 1) which together accounted for 60.8% of the variation in feeding activity were retrieved (Table 2). PC1 (39.3% of variation) described feeding activity at the late ontogenetic stage, while PC2 (21.5% of variation) described feeding activity at the early ontogenetic stage (Table 2). GLMMs of the two PCs revealed significant interactions between origin of father and origin of mother in both cases (Table 3). Family effect was significant for the early phase ($Z = -3.01$, $P = 0.003$) but not for the late phase ($Z = 0.56$, $P = 0.57$). Tukey–Kramer tests showed that in early behaviour, HEL-HEL fish differed from all other crosses (all $P < 0.0008$), while the other crosses did not differ (all $P > 0.19$; Fig. 2a). With respect to the behaviour at the later ontogenetic stage, the picture was a bit more complex; PYÖ-PYÖ fish differed from all other crosses (all $P < 0.013$), HEL-PYÖ also differed from all other crosses (all $P < 0.034$), while HEL-HEL and PYÖ-HEL were indistinguishable ($P = 0.99$; Fig. 2b). Early in the ontogenesis, PYÖ-PYÖ, HEL-PYÖ and PYÖ-HEL showed higher feeding activity than HEL-HEL, while in the later stage, HEL-HEL, HEL-PYÖ and PYÖ-HEL were all less active feeders than PYÖ-PYÖ, HEL-PYÖ being more active than HEL-HEL and PYÖ-HEL. In both stages, the patterns were indicative of nonadditive genetic effects, namely dominance. In the early phase, the pond fish expressed a dominant pattern for high feeding activity, while in the later phase, marine fish expressed a dominant pattern for low feeding activity. In the late phase, the divergence of HEL-PYÖ from HEL-HEL and PYÖ-HEL suggests also possible influence of maternal effects exerted by pond mothers (Figs 1 and 2).

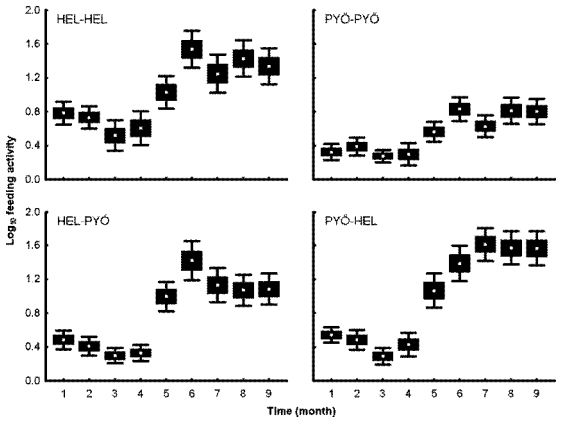


Fig. 1 Mean [± standard error (boxes) + 95% confidence intervals (whiskers)] feeding activities as a function of time showing ontogenetic shifts until 9 months after hatching in pure marine (HEL-HEL), pure pond (PYÖ-PYÖ) and marine-pond hybrid nine-spined stickleback crosses with marine father – pond mother (HEL-PYÖ) and pond father – marine mother (PYÖ-HEL). Low values represent high feeding activity (short latency to feed).

Table 2 Results of the principal component analysis of the nine feeding activity measures. Factor loadings of principal components (PC) with eigenvalues > 1 are shown.%var = percentage of variance explained.

Measure	PC1	PC2
Month 1	0.41	0.60
Month 2	0.40	0.54
Month 3	0.49	0.62
Month 4	0.52	0.53
Month 5	0.50	0.13
Month 6	0.43	0.06
Month 7	0.86	–0.42
Month 8	0.88	–0.43
Month 9	0.86	–0.46
% var	39.35	21.47
Eigenvalue	3.54	1.93

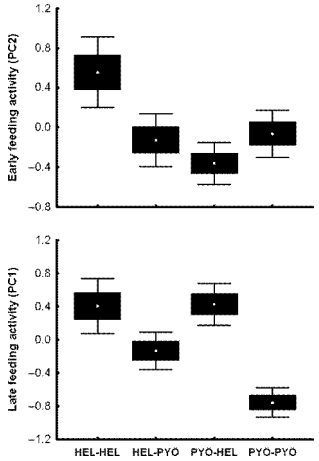


Fig. 2 Differences among pure marine (HEL-HEL), pure pond (PYÖ-PYÖ) and hybrid nine-spined stickleback crosses with marine father – pond mother (HEL-PYÖ) or pond father – marine mother (PYÖ-HEL) in feeding activity during the early [a; represented by principal component (PC) 2] and late (b; represented by PC1) life stages. Means ± standard error (boxes) + 95% confidence intervals (whiskers) are shown. Low values represent high feeding activity (short latency to feed).

Discussion

Geographical variation in behaviour is well documented (Foster, 1999; Foster & Endler, 1999). Variation in predation risk is one of the best studied biotic environmental factors shaping behaviour (e.g. Giles & Huntingford, 1984; Riechert & Hedrick, 1990; Mathis *et al.*, 1993; Huntingford *et al.*, 1994; Storfer & Sih, 1998; Åbjörnsson *et al.*, 2004). The effect of predation is often seen in a wide variety of behavioural traits, for instance, boldness, sociability and aggression all covary with predation risk across guppy (*Poecilia reticulata*) populations (Magurran & Seghers, 1991, 1994). In our study system, pond sticklebacks are more aggressive, bolder/more risk-taking, more explorative and have higher feeding activity than their marine conspecifics (Herczeg *et al.*, 2009b; Herczeg & Välimäki, 2011). Feeding activity might be a particularly important behavioural trait in the nine-spined stickleback adaptive divergence, given that pond sticklebacks have repeatedly turned into giants (Herczeg *et al.*, 2009a, 2010a) with an extended period of growth (Herczeg *et al.*, 2012), and delayed maturation (Shimada *et al.*, 2011; Ab Ghani *et al.*, 2013). Hence, high feeding activity must be an important adaptation in ponds in the absence of piscine predators where intraspecific competition seems to be the most relevant process affecting fitness (e.g. Herczeg *et al.*, 2012). However, there is obviously variation within populations too, which cannot be explained by behavioural plasticity or measurement error, and this variation (i.e. animal personality) has important evolutionary implications (e.g. Wilson, 1998; Dingemanse *et al.*, 2010). Here, we showed that nine-spined sticklebacks do have 'feeding personalities', and further, the individual component in behaviour can be extended along ontogeny, translating to individual personality shifts between different ages. Feeding activity is not identified as a personality trait itself (*sensu* Réale *et al.*, 2007), but we believe it is an important behaviour in our system (see above). Therefore, as animal personality refers to individual consistency in behaviour (e.g. Sih & Bell, 2008), and feeding activity was consistent between individuals, we believe that usage of the term

Table 3 Results from the General Linear Mixed Models of the principal components separating feeding activity in the early and late life stages. For details on the principal component analysis, see Table 2 and text. Fixed effects are shown here, random effects are reported in the text.

Effect	ndf	ddf	*F*	*P*
Early life stage				
Origin of father (F)	1	37.3	17.46	0.0002
Origin of mother (M)	1	37.3	3.64	0.06
F × M	1	37.3	23.23	< 0.0001
Late life stage				
Origin of father (F)	1	36.7	4.94	0.032
Origin of mother (M)	1	36.7	40.85	< 0.0001
F × M	1	36.7	5.66	0.023

ndf, denotes the numerator.
ddf, the denominator degrees of freedom.

'feeding personality' here is justified. Similar terms could be used about any other consistent behaviour not directly classifiable following Réale *et al.* (2007). At any rate, feeding activity has been found to be positively correlated with risk-taking, that is, feeding activity following a simulated predatory attack (Herczeg *et al.*, 2009b). Thus, it is a good descriptor of an internal 'drive to feed' under various conditions.

The variation in ontogenetic feeding personalities was affected by several factors. First, we observed a general trend – irrespective of within and between population variation – of decreasing feeding activity along ontogeny. Activity in general, including foraging activity, increases the probability of an encounter with a predator (e.g. Lima & Dill, 1990). On the other hand, when faced with gape-limited predators, fast growth (and thus high foraging activity) is advantageous for the prey because it can reach a critical size above which the predator is not dangerous anymore (Werner & Gilliam, 1984; Abrams & Rowe, 1996; Urban, 2008). Therefore, in the early stages when fry are extremely small and vulnerable to many predators (cannibalism and insect predation are relevant dangers both in ponds and marine habitats) aiming for a size-refuge from many size-restricted predators should be a good strategy. Later on, when drastic size changes are not possible due to developmental/phylogenetic constraints, decreasing activity might be a better antipredator strategy. However, while there was a general decrease in activity with age, there were also marked differences between the crosses, the most striking being that between the pure crosses: marine fish were less active than pond fish during the whole observation period, but also, they showed a much stronger decrease in their activity with age. In previous studies, we have found that Pyöreälampi and Helsinki fish express different growth strategies (Shimada *et al.*, 2011; Herczeg *et al.*, 2012): while Helsinki fish grow to small final size and reach that size quickly, Pyöreälampi fish grow to large final size but reach it slowly. Helsinki fish are sympatric to a large number of gape-unlimited predatory fish species, and thus have to trade-off growth with survival, and reproduce as soon as possible (at small size), while Pyöreälampi fish face low predation risk as adults, and hence, they grow to as large as possible to gain competitive advantage in intraspecific interactions (for more details, see Herczeg *et al.*, 2009a, 2012). The growth strategy divergence is fully congruent with what we found here in terms of feeding activity divergence, and both observations can be explained by the differences in mortality risk caused by (piscine) predation (Roff, 1992; Stearns, 1992).

The pattern of divergence between the four experimental crosses was complex as indicated by the three-way interaction between origin of father, origin of mother and fish age. This suggests that the divergence in the ontogenetic shift in feeding personalities has a genetic basis, but also that it cannot be explained by simple additive genetic effects alone (cf. Wright 1978; Lynch & Walsh, 1998; Laugen *et al.*, 2002). When focussing on the ontogenetic pattern, we found that ontogenetic feeding activity can be separated into two main independent components: early (months 1–4) and late feeding activity (months 6–9). The analyses of these components revealed interactions between the origins of father and mother in both cases, again suggesting that an additive genetic scenario alone is not satisfactory. By comparing the hybrids to the pure crosses, an interesting pattern emerged: in both cases a nonadditive genetic scenario (i.e. dominance) seemed to best explain the data, because in both cases, the hybrids grouped with one of the pure crosses, while the other pure cross was clearly divergent. The interesting finding was that the hybrids were similar to different pure crosses in the early and late ontogenetic stages, respectively. Altogether, the patterns suggest that in the early phase, the high feeding activity of pond sticklebacks, while in the late phase, the low feeding activity of marine sticklebacks was dominant. Hence, assuming that our coastal marine population represents the ancestral form, after nine-spined stickleback became isolated in Pyöreälampi, it evolved a dominant character (high feeding activity as young fish) and also managed to overcome an ancestral dominant character (low feeding activity as adult fish).

We detected some signs of possible maternal effects in late feeding activity as well. The effect was asymmetric; it was only found in hybrids with pond mothers. It seems that in the isolated pond, both genetic and maternal effects favour high feeding activity at all life stages. Maternal effects are widely acknowledged as important sources of phenotypic variation (Kaplan, 1998; Mousseau & Fox, 1998). They can be mediated via environment or maternal resources (Lacey, 1998; Mousseau & Fox, 1998; Rossiter, 1998; Laugen *et al.*, 2002; Green, 2008), and can persist through several generations (Lacey, 1998; Rossiter, 1998; Green, 2008). In our case, where offspring was produced *in vitro*, pond mothers could only affect their offspring by their eggs' composition (i.e. reflecting maternal quality and/or maternal investment) or by heritable nongenetic effects. To completely remove maternal and environmental effects, or fully control for them, several laboratory generations and/or complex breeding designs would be necessary (e.g. Lynch & Walsh, 1998). However, the patterns were quite robust in our case, making undetected environmental effects as important sources of variation unlikely.

In summary, we found consistent 'feeding personalities' across different populations and their different hybrids, and further, individually consistent ontogenetic shifts in that feeding personality. There is an emerging interest towards the developmental approach in studying animal personality (e.g. Rodel & Monclus, 2011;

Trillmich & Hudson, 2011) and our study provides an example how personality changes with time and how personality at different life stages can have different genetic background. The strong population divergence between our coastal marine and isolated pond populations could be explained by dominant genetic effects and an asymmetric maternal effect. The ontogenetic patterns are consistent with a scenario where different populations seem to harbour the dominant allele(s) in different life stages. Therefore, it seems that pond stickleback evolved a dominant character in their early life stage and got over a dominant marine character in their later life stage. This, together with a possible maternal effect transmitted only by pond females, ensure that pond stickleback have much higher feeding activity throughout their lifespan, which might be necessary for reaching the giant sizes observed in several independent ponds.

Acknowledgments

We are highly indebted for Riku Paavola, Pirko Siikamäki and the Oulanka Research Station for their inevitable support during the fieldwork. We thank John Loehr for correcting the English. The project received financial support from the Finnish Academy (# 250435 for Juha Merilä, and 128716 for Gábor Herczeg) and Universiti Putra Malaysia for Nurul Izza Ab Ghani.

References

Ab Ghani, N.I., Herczeg, G. & Merilä, J. 2012. Body size divergence in nine-spined sticklebacks: disentangling additive genetic and maternal effects. *Biol. J. Linn. Soc.* **107**: 521–528.

Ab Ghani, N.I., Herczeg, G., Leinonen, T. & Merilä, J. 2013. Evidence for genetic differentiation in timing of maturation among nine-spined stickleback populations. *J. Evol. Biol.* (in press), doi:10.1111/jeb.12085.

Åbjörnsson, K., Hansson, L.A. & Brönmark, C. 2004. Responses of prey from habitats with different predator regimes: local adaptation and heritability. *Ecology* **85**: 1859–1866.

Abrams, P.A. & Rowe, L. 1996. The effects of predation on the age and size of maturity of prey. *Evolution* **50**: 1052–1061.

Bănărescu, P.M. & Paepke, H.-J. 2001. *The Freshwater Fishes of Europe*, vol. **5/III**. AULAVerlag, Wiebelsheim, Germany.

Bell, A.M. 2007. Future directions in behavioural syndrome research. *Proc. Biol. Sci.* **274**: 755–761.

Dingemanse, N.J., Both, C., Drent, P.J., van Oers, K. & van Noordwijk, A.J. 2002. Repeatability and heritability of exploratory behaviour in great tits from the wild. *Anim. Behav* **64**: 929–938.

Dingemanse, N.J., Van der Plas, F., Wright, J., Réale, D., Schrama, M., Roff, D.A. *et al.* 2009. Individual experience and evolutionary history of predation affects expression of heritable variation in fish personality and morphology. *Proc. Biol. Sci.* **276**: 1285–1293.

Dingemanse, N.J., Kazem, A.J.N., Réale, D. & Wright, J. 2010. Behavioural reaction norms: where animal personality meets individual plasticity. *Trends Ecol. Evol.* **25**: 81–89.

Dingemanse, N.J., Barber, I., Wright, J. & Brommer, J.E. 2012. Quantitative genetics of behavioural reaction norms: genetic correlations between personality and behavioural plasticity vary across stickleback populations. *J. Evol. Biol.* **25**: 485–496.

Drent, P.J., van Oers, K. & van Noordwijk, A.J. 2003. Realized heritability of personalities in the great tit (*Parus major*). *Proc. R. Soc. Lond. B* **270**: 45–51.

Foster, S.A. 1999. The geography of behaviour: an evolutionary perspective. *Trends Ecol. Evol.* **14**: 190–195.

Foster, S.A. & Endler, J.A. 1999. *Geographic Variation in Behavior: Perspectives on Evolutionary Mechanisms*. Oxford University Press, New York.

Garamszegi, L.Z., Markó, G. & Herczeg, G. 2012. A meta-analysis of correlated behaviours with implications for behavioural syndromes: mean effect size, publication bias, phylogenetic effects and the role of mediator variables. *Evol. Ecol.* **26**: 1213–1235.

Giles, N. & Huntingford, F.A. 1984. Predation risk and interpopulation variation in anti-predator behaviour in the three-spined stickleback, *Gasterosteus aculeatus* L. *Anim. Behav.* **32**: 264–275.

Gosling, S.D. 2001. From mice to men: what can we learn about personality from animal research. *Psychol. Bull.* **127**: 45–86.

Green, B.S. 2008. Maternal effects in fish populations. *Adv. Mar. Biol.* **54**: 1–105.

Herczeg, G. & Välimäki, K. 2011. Intraspecific variation in behaviour: effects of evolutionary history, ontogenetic experience and sex. *J. Evol. Biol.* **24**: 2434–2444.

Herczeg, G., Gonda, A. & Merilä, J. 2009a. Evolution of gigantism in nine-spined sticklebacks. *Evolution* **63**: 3190–3200.

Herczeg, G., Gonda, A. & Merilä, J. 2009b. Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *J. Evol. Biol.* **22**: 544–552.

Herczeg, G., Gonda, A. & Merilä, J. 2010a. Rensch's rule inverted – female-driven gigantism in nine-spined stickleback (*Pungitius pungitius*). *J. Anim. Ecol.* **79**: 581–588.

Herczeg, G., Turtiainen, M. & Merilä, J. 2010b. Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. *Biol. J. Linn. Soc.* **101**: 413–416.

Herczeg, G., Gonda, A., Kuparinen, A. & Merilä, J. 2012. Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? *Evol. Ecol.* **26**: 109–122.

Huntingford, F.A., Wright, P.J. & Tierney, J.F. 1994. Adaptive variation in antipredator behaviour in threespine stickleback. In: *The Evolutionary Biology of Threespine Stickleback* (M.A. Bell & S.A. Foster, eds), pp. 277–296. Oxford University Press, New York.

Kahilainen, K., Malinen, T., Tuomaala, A. & Lehtonen, H. 2004. Diel and seasonal habitat and food segregation of three sympatric *Coregonus lavaretus* forms in a subarctic lake. *J. Fish Biol.* **64**: 418–434.

Kaplan, R.H. 1998. Maternal effects, developmental plasticity and life history evolution. In: *Maternal Effects as Adaptations* (T.A. Mousseau & C.W. Fox, eds), pp. 244–260. Oxford University Press, New York.

Lacey, E.P. 1998. What is an environmentally induced parental effect? In: *Maternal Effects as Adaptations* (T.A. Mousseau & C.W. Fox, eds), pp. 54–66. Oxford University Press, New York.

Laugen, A.T., Laurila, A. & Merilä, J. 2002. Maternal and genetic contributions to geographical variation in *Rana temporaria* larval life-history traits. *Biol. J. Linn. Soc.* **76**: 61–70.

Lima, S.L. & Dill, L.M. 1990. Behavioural decisions made under the risk of predation – a review and prospectus. *Can. J. Zool.* **68**: 619–640.

Littell, R.C., Rendergast, J. & Natarajan, R. 2000. Modeling covariance structure in the analysis of repeated measures data. *Stat. Med.* **19**: 1793–1819.

Littell, R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D. & Schabenberger, O. 2006. *SAS for Mixed Models*, 2nd edn. SAS Institute Inc., Cary, NC.

Lynch, M. & Walsh, B. 1998. *Genetics and Analysis of Quantitative Traits*. Sinauer Associates, Sunderland, MA.

Magurran, A.E. & Seghers, B.H. 1991. Variation in schooling and aggression amongst guppy (*Poecilia reticulata*) populations in Trinidad. *Behaviour* **118**: 214–234.

Magurran, A.E. & Seghers, B.H. 1994. Predator inspection behaviour covaries with schooling tendency amongst wild guppy, *Poecilia reticulata*. *Behaviour* **128**: 121–134.

Mathis, A., Chivers, D.P. & Smith, R.J.F. 1993. Population differences in responses of fathead minnows (*Pimephales promelas*) to visual and chemical stimuli from predators. *Ethology* **93**: 31–40.

Mousseau, T.A. & Fox, C.W. 1998. *Maternal Effects as Adaptations*. Oxford University Press, New York.

van Oers, K., Drent, P.J., de Jong, G. & van Noordwijk, A.J. 2004. Additive and nonadditive genetic variation in avian personality traits. *Heredity* **93**: 496–503.

Östlund-Nilsson, S., Mayer, I. & Huntingford, F.A. 2007. *Biology of the Three-spined Stickleback*. CRC Press, Boca Raton, FL.

Réale, D., Reader, S.M., Sol, D., McDougall, P.T. & Dingemanse, N.J. 2007. Integrating animal temperament within ecology and evolution. *Biol. Rev.* **82**: 291–318.

Riechert, S.E. & Hedrick, A.V. 1990. Levels of predation and genetically based antipredator behaviour in the spider, *Agelenopsis aperta*. *Anim. Behav.* **40**: 679–687.

Rodel, H.G. & Monclus, R. 2011. Long-term consequences of early development on personality traits: a study in European rabbits. *Behav. Ecol.* **22**: 1123–1130.

Roff, D.A. 1992. *The Evolution of Life Histories*. Chapman & Hall, New York.

Rossiter, M. 1998. The role of environmental variation in parental effects expression. In: *Maternal Effects as Adaptations* (T.A. Mousseau & C.W. Fox, eds), pp. 112–134. Oxford University Press, New York.

Shikano, T., Shimada, Y., Herczeg, G. & Merilä, J. 2010. History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. *Mol. Ecol.* **19**: 1147–1161.

Shimada, Y., Shikano, T., Kuparinen, A., Gonda, A., Leinonen, T. & Merilä, J. 2011. Quantitative genetics of body size and timing of maturation in two-nine-spined stickleback (*Pungitius pungitius*) populations. *PLoS ONE* **6**: e28859.

Sih, A. & Bell, A.M. 2008. Insights from behavioral syndromes for behavioral ecology. *Adv. Study Behav.* **38**: 277–281.

Sih, A., Bell, A.M. & Johnson, J.C. 2004a. Behavioural syndromes: an ecological and evolutionary overview. *Trends Ecol. Evol.* **19**: 372–378.

Sih, A., Bell, A.M., Johnson, J.C. & Ziemba, R.E. 2004b. Behavioral syndromes: an integrative overview. *Q. Rev. Biol.* **79**: 241–277.

Smith, B.R. & Blumstein, D.T. 2008. Fitness consequences of personality: a meta-analysis. *Behav. Ecol.* **19**: 448–455.

Stearns, S.C. 1992. *The Evolution of Life Histories*. Oxford University Press, New York.

Storfer, A. & Sih, A. 1998. Gene flow and ineffective antipredatory behavior in a stream-breeding salamander. *Evolution* **52**: 558–565.

Trillmich, F. & Hudson, R. 2011. The emergence of personality of animals: the need for a developmental approach. *Dev. Psychobiol.* **53**: 505–509.

Urban, M.C. 2008. Salamander evolution across a latitudinal cline in gape-limited predation risk. *Oikos* **117**: 1037–1049.

Välimäki, K. & Herczeg, G. 2012. Ontogenetic and evolutionary effects of predation and competition on nine-spined stickleback (*Pungitius pungitius*) body size. *J. Anim. Ecol.* **81**: 859–867.

Werner, E.E. & Gilliam, J.F. 1984. The ontogenetic niche and species interactions in size structured populations. *Annu. Rev. Ecol. Syst.* **15**: 393–425.

Wilson, D.S. 1998. Adaptive individual differences within single populations. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **353**: 199–205.

Wright, S. 1978. Variability within and among populations. In: *Evolution and the genetics of populations* (S. Wright, ed.), pp. 336–347. University Chicago Press, Chicago, IL.

Received 25 July 2012; revised 13 November 2012; accepted 10 December 2012

Evol Ecol (2012) 26:109–122
DOI 10.1007/s10682-011-9491-9

ORIGINAL PAPER

Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition?

Gábor Herczeg · Abigél Gonda · Anna Kuparinen · Juha Merilä

Received: 2 February 2011 / Accepted: 17 May 2011 / Published online: 27 May 2011

Abstract Gigantism in isolated ponds in the absence of sympatric fish species has previously been observed in nine-spined sticklebacks (*Pungitius pungitius*). Patterns in sexual size dimorphism suggested that fecundity selection acting on females might be responsible for the phenomenon. However, the growth strategy behind gigantism in pond sticklebacks has not been studied yet. Here, we compared von Bertalanffy growth parameters of four independent nine-spined stickleback populations reared in a common laboratory environment: two coastal marine (typical size) and two pond (giant size) populations. We found that both pond populations had larger estimated final size than marine populations, which in turn exhibited higher intrinsic growth rates than the pond populations. Female growth strategies were more divergent among marine and pond populations than those of males. Asymptotic body size and intrinsic growth rate were strongly negatively correlated. Hence, pond versus marine populations exhibited different growth strategies along a continuum. Our data suggest that quick maturation—even with the cost of being small (low fecundity)—is favoured in marine environments. On the contrary, growth to a giant final size (high fecundity)—even if it entails extended growth period—is favoured in ponds. We suggest that the absence (ponds) versus presence (marine environment) of sympatric predatory fish species, and the consequent change in the importance of intraspecific competition are responsible for the divergence in growth strategies. The sex-dependence of the patterns further emphasizes the role of females in the body size divergence in the species. Possible alternative hypotheses are also discussed.

Keywords Adaptive divergence · Body size · Growth rate · Life history · Natural selection · Predation

G. Herczeg (✉) · A. Gonda · A. Kuparinen · J. Merilä
Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, PO Box 65, 00014 Helsinki, Finland
e-mail: gabor.herczeg@helsinki.fi

Introduction

Body size is a trait of paramount ecological and evolutionary significance, and often correlates with other traits important for physiology and fitness (Peters 1983; Roff 1992; Stearns 1992). Different forms of sexual selection (mate choice, male-male competition, fecundity selection) usually favour larger body size (Shine 1989; Andersson 1994). The fact that evolution towards larger body size is not taking place in many macro- and microevolutionary contexts suggests that there must be selective forces acting against large body size (e.g. Blanckenhorn 2000). Two general factors selecting against large body size are viability selection induced by size-unlimited predators selecting larger individuals (e.g. Blanckenhorn 2000), and interspecific competition where different species competing for overlapping resources stabilize their 'size niches' (Wilson 1975; Lomolino 1985; Simberloff et al. 2000). Not only large body size, but reaching it quickly can be also advantageous, as it prolongs the time available for reproduction by decreasing the time over which resources are devoted exclusively to somatic growth (Sibly and Calow 1986; Blanckenhorn and Demont 2004). Furthermore, fast growth can be advantageous also if it helps to reach some critical size beyond which predation pressure by size-limited predators becomes relaxed (Werner and Gilliam 1984; Abrams and Rowe 1996; Urban 2008). Despite these potential benefits, most organisms in nature experience growth rates below their maximal physiological capacity (e.g. Calow 1982; Atchley 1984). The main factors selecting against faster growth are resource limitation, and increased susceptibility of active foragers to gape-unlimited (i.e. stage- or size-unlimited) predation (e.g. Lima and Dill 1990; Abrams and Rowe 1996; Day and Rowe 2002; Biro et al. 2004, 2006).

An evolutionary shift in body size can be reached by altering either the length of growth period and/or rate of growth. Interestingly, a negative correlation between growth rate and asymptotic size is often found in fish (Berrigan and Charnov 1994), suggesting the presence of different growth strategies: being small but growing quickly, or being large and growing slowly (relative to the final size). Such strategies can be also understood through the hypothesis of optimal energy allocation; energy surplus to that required for maintenance can be allocated to somatic growth and reproduction (e.g. Roff 1992; Stearns 1992). The optimal allocation strategy will depend on the selective environment (Heino and Kaitala 1999), including both abiotic (e.g. temperature) and biotic (e.g. predation) factors. However, while growth rate comparisons between populations along environmental clines are relatively abundant, they are scarce between populations differing in predation risk (Dmitriew 2011). As a rare example, Arendt and Reznick (2005) compared growth patterns of low size-limited predation risk/low resource availability guppy (*Poecilia reticulata* Peters, 1859) populations to those of high size-unlimited predation risk/high resource availability populations, and concluded that resource availability is a more important factor in shaping growth evolution than the different predator regimes in their system.

The nine-spined stickleback (*Pungitius pungitius* Linneaeus, 1758) is a small teleost inhabiting a wide array of habitats. It can be found in coastal marine environments, large lakes and river systems, small creeks and ditches, and also in isolated ponds (Bănărescu and Paepke 2001; Östlund-Nilsson et al. 2007). Nine-spined sticklebacks in ponds have evolved into giants as compared to typical-sized coastal marine (or lake) populations (Herczeg et al. 2009a, 2010a). Marked differences between pond and coastal marine populations—apart from the size of the habitats—can be found: (i) nine-spined stickleback is the only fish species in ponds, while it is a member of a diverse fish fauna in coastal marine environments and (ii) salinity and the ion concentrations (e.g. calcium) are different between seawater and freshwater. The fact that sticklebacks are the only fish species

present in many ponds suggests two things. First, predation pressure posed by piscine predators is zero in ponds, and second, intraspecific competition for resources in the supposedly low-productivity pond environments might be the main biotic environmental factor affecting fitness. Salinity/ion concentrations are also known to affect stickleback development. In the tree-spined stickleback (*Gasterosteus aculeatus*), morphs with reduced bony armour had a growth advantage in freshwater over their heavily armoured conspecifics (Marchinko and Schluter 2007; Barrett et al. 2008), probably due to the high costs of bone mineralization in freshwater (Giles 1983; Bell et al. 1993). Hence, predation, competition and salinity are all possible—nonexclusive—drivers of the body size divergence between nine-spined stickleback populations. However, considering that freshwater nine-spined sticklebacks from lake populations are similarly sized and armoured to coastal marine sticklebacks, but highly divergent in both size and bony armour from pond sticklebacks (Herczeg et al. 2009a, 2010a, b), the most likely explanation for the evolution of the extraordinary pond phenotype appears to be the lack of piscine predation coupled with high intraspecific competition. This conjecture is further strengthened by the facts that pond nine-spined sticklebacks are bolder and more aggressive (Herczeg et al. 2009b), and facing an increased cost of group living (Gonda et al. 2009; Herczeg et al. 2009c) when compared to their marine conspecifics.

In this study, we provide a detailed comparison of growth strategies of giant pond and typical-sized coastal marine nine-spined stickleback populations to identify and quantify the growth strategy that leads to giant-sized individuals in this species. In theory, larger body size can be achieved by higher growth rate or/and an extended growth period. However, growth rate and final body size is usually negatively correlated (Berrigan and Charnov 1994), suggesting different growth strategies. We hypothesised that marine fish grow quickly to a small final body size while pond sticklebacks grow slowly to a giant body size. As our previous findings suggest that females drive the size divergence between habitats (Herczeg et al. 2010a), we also expected females to show larger divergence in growth strategy than males. We also aimed to provide an example of growth strategy divergence between populations that are likely to differ in predation pressure (complete lack of sympatric predatory fishes in ponds versus diverse sympatric predatory fish fauna in the coastal marine sites) because studies to this effect are rare (Dmitriew 2011; but see Arendt and Reznick 2005). We tested our predictions by studying common garden growth data (from hatching to approaching the final size) of geographically and genetically independent coastal marine and pond populations. In addition, we were interested to see if the among-population body size variation in the wild reflects genetic differences in final size as inferred from comparisons of data from the wild and laboratory.

Materials and methods

Field sampling, breeding, rearing and measurements

We sampled two marine (Baltic and White Sea) and two pond (Bynästjärnen in Sweden, and Pyöreälampi in Finland) populations, all of which were both geographically (Fig. 1) and genetically (Shikano et al. 2010) isolated. The marine sites were shallow coastal bays close to creek inlets, and thus, they represented low salinity sea habitats (Baltic Sea being a brackish water environment in general). In marine habitats, nine-spined sticklebacks belong to a diverse fish community including a large number of predator species. Conversely, ponds lack sympatric fish. In Pyöreälampi, a few, small whitefish (*Coregonus*

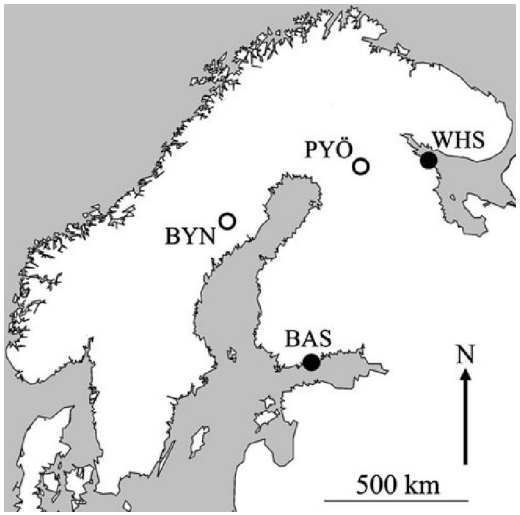


Fig. 1 Map of Fennoscandia showing the sampling localities. *Filled circles* denote marine, *open circles* pond populations. *BAS* Baltic Sea, Finland; *WHS* White Sea, Russia; *BYN* Bynästjärnen (pond, Sweden); and *PYÖ* Pyöreälampi (pond, Finland)

lavaretus Linnaeus 1758) were recently introduced, but the dietary habits of the species (Kahilainen et al. 2004) make it more of a competitor than a predator of sticklebacks. Predation by aquatic insects and cannibalism might be relevant at all sites for early life stages of sticklebacks. Due to the relatively small surface area, the ponds do not host permanent piscivorous birds (we never observed one during our extensive field work). However, the effects of some sporadic visiting birds cannot be excluded. At any rate, bird predation in ponds is unlikely to be higher than in the coastal marine sites. There are two facts that indicate relaxed predation in ponds as compared to the marine sites: first, nine-spined sticklebacks have undergone strong reduction in their defensive armour in ponds (reduction or even loss of the pelvic apparatus; Herczeg et al. 2010b), and second, pond sticklebacks live ca. two times longer in nature than marine (or large lake) sticklebacks (Herczeg et al. 2009a).

Detailed descriptions of the procedures used for the common garden experiment are available in Herczeg et al. (2009b, c). Briefly, adult sticklebacks were collected during the reproductive season of 2007, and transferred to the aquacultural facilities of the University of Helsinki. Crosses (5 full-sib families from each population) were done in vitro. The fertilized clutches were transferred to 1.4 l tanks of Allentown Zebrafish Rack Systems (hereafter rack, Aquaneering Inc., San Diego, USA). Racks were equipped with physical, chemical, biological and UV filters. Four days after hatching, when fry started to swim freely but not yet feeding, 10 fry from every family were randomly distributed among the 200 1.4 l tanks (4 populations × 5 families × 10 individuals) of two racks. Extra fish were kept separately for other purposes. Water temperature was set to 17°C, and visual stimuli between tanks were blocked by white plastic panels. Fish were first fed with live brine shrimp (*Artemia* sp.) nauplii, then with frozen copepods (*Cyclops* sp.) and bloodworms

(Chironomidae larvae) twice a day in excess. A 24 h light photoperiod (representative of summer conditions at high latitudes) was used until the fish were 12 weeks old, after which it was gradually changed to a 12:12 h light:dark photoperiod over the course of 1 week. Because of the latitudinal differences between populations, we did not aim to mimic natural light conditions better.

Fish were first photographed 4 days after hatching, before individual rearing was started. Later on, each individual fish was photographed at the following intervals after the first photographing on day 4: +1 week, +1 week, +2 weeks, +2 weeks, +3 weeks, +3 weeks, +4 weeks, +4 weeks, +8 weeks, +8 weeks (=36 weeks); the last photographs were taken on day 256 after hatching. Digital photographs were taken with a Panasonic DMC-FZ8 digital camera on a tripod. As the fish grew, we had to change the camera-to-fish distance (it was fixed for a given round of measurement), but all photographs were taken under standardized settings and a millimetre scale was placed in every photograph for scaling. Standard length (from the tip of the nose to the end of the tail base) was measured from the photographs by using of tps. Dig 2.10 (Rohlf 2006) software. Because of mortality (affected fish at random) and other scientific purposes (fish chosen randomly, but affecting all families evenly) the final growth dataset included 86 fish; 21 from the Baltic Sea (family representations: 6, 5, 4, 3, 3), 23 from the White Sea (family representations: 7, 6, 5, 3, 2), 20 from Bynastjärnen (family representations: 7, 4, 3, 3, 3) and 22 from Pyörälampi (family representations: 6, 5, 5, 5, 1). Sex was identified by dissection and gonadal inspection at the end of the experiment. We note that although using F1 laboratory generation should remove a large proportion of environmental variation, some maternal effects and/or cross-generational influences may remain. However, most maternal effects on growth dissipate quickly and seldom explain any large interpopulation differences in growth (e.g. Green 2008).

Statistical analyses

Each individual growth trajectory (based on 11 measurements, see above) was summarized through a von Bertalanffy growth curve (von Bertalanffy 1938), which is commonly applied to similar data (e.g. Katsanevakis 2006; Kuparinen et al. 2011) and takes the functional form:

$$l(t) = L_{\max} - (L_{\max} - L_0)e^{-kt} \quad (1)$$

where l(t) is the length of an individual at age t, $L_{\max}$ is the asymptotic length (hereafter estimated final length), k is the growth constant (hereafter intrinsic growth rate), and L_0 estimates length at $t = 0$ (no biological relevance in this case). Note that k is not a simple unit size change/unit time growth rate, but an intrinsic characteristic of the growth curve, describing how quickly the curve saturates. This approach provides a far better understanding of the growth strategy than the simple estimation of growth rate using size and developmental time could, because the latter would be 1:1 related to the size measured at the end of this experiment with fixed time period. The curve (1) was fitted to the length-at-age measurements through non-linear least-squares regression. Therefore, despite their mechanistic interpretation, k, L_0 and $L_{\max}$ were treated as free model parameters. The von Bertalanffy curves fit the individual trajectories very well (R^2 range: 0.977–0.998), and hence, the derived parameters ($L_{\max}$, k) described individual growth with high accuracy.

The analyses of $L_{\max}$ and k were done at three levels. First, we ran General Linear Mixed Models (GLMMs) with habitat type (marine vs. pond) as fixed, and population

nested in habitat and family nested in population as random factors to directly test for habitat effects. Second, we ran GLMMs with population as fixed and family nested in population as random factors to be able to compare populations pairwise with the aid of Bonferroni post hoc tests. Third, we ran a GLMM with k as a dependent variable, $L_{\max}$ as covariate, population as fixed, and family nested within population as random factor to test for a possible correlation between the two growth parameters. Because the two variables indeed correlated (see "Results"), we also ran a Principal Component Analysis (PCA) on them to collapse them into a single variable. Then, using the PC scores as dependent variable, we ran GLMMs at both habitat and population levels as outlined above. Unfortunately, there were only two males in the Bynästjärnen sample, making analysis of sex in the above models impossible. However, to explore if there were any sex effects on the patterns, we fitted two GLMMs to data that was restricted to the three populations with representative samples of both sexes. Here, $L_{\max}$ or k were dependent variables, population, sex, and their interaction fixed factors, and family nested within population a random factor.

Finally, we compared the length of the largest wild-caught fish from a given population to the mean asymptotic length estimate of that population from our experiment, using one-sample t-tests. In an attempt to control for the potential bias of outliers, we ran the same tests using the mean size of the five largest fish from a population instead of the size of the largest fish, but the results remained qualitatively similar (data not shown). Analyses were carried out with R 2.10.1 (R development Core Team 2009) and PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA).

Results

The habitat-level GLMM on $L_{\max}$ revealed a significant habitat effect, pond fish had larger estimated final length than marine fish ($F_{1,\ 4.03} = 20.74$; $P = 0.01$; Least Squares [LS] mean ± Standard Error [SE]: marine = 56.87 mm ± 4.12; pond = 83.44 mm ± 4.13). The population effect was nonsignificant (Z = 1.06; $P = 0.29$) while the family effect approached significance (Z = 1.80; $P = 0.07$). The population level GLMM revealed a significant population effect ($F_{3,\ 17.82} = 43.28$; $P < 0.001$, Fig. 2a). The family effect approached significance (Z = 1.77; $P = 0.08$). Apart from the two pond populations ($P > 0.99$), all other pairs differed in $L_{\max}$ (all $P < 0.002$, Fig. 2a).

The habitat-level GLMM on k revealed a significant habitat effect, marine fish having higher intrinsic growth rate than pond fish ($F_{1,\ 3.96} = 24.32$; $P = 0.008$; LS mean ± SE: marine = 0.13 ± 0.001; pond = 0.008 ± 0.001). The population effect was nonsignificant (Z = 0.54; $P = 0.59$) while the family effect approached significance (Z = 1.91; $P = 0.06$). The population level GLMM revealed a significant population effect ($F_{3,\ 18.43} = 22.33$; $P < 0.001$, Fig. 2b). The family effect approached significance (Z = 1.88; $P = 0.06$). Apart from the two marine populations ($P > 0.99$), all other pairs of population differed from each other (all $P < 0.03$, Fig. 2b).

The third GLMM revealed that k was significantly related to $L_{\max}$, irrespective of population origin (population: $F_{3,\ 68.40} = 2.78$; $P = 0.048$; $L_{\max}$: $F_{1,\ 69.75} = 2.78$; $P < 0.001$; population × $L_{\max}$: $F_{3,\ 70.21} = 1.59$; $P = 0.20$). Family effect was nonsignificant (Z = 1.18; $P = 0.24$). The overall correlation was strong and negative ($r_{86} = -0.85$, $P < 0.001$; Fig. 3). The PCA on k and $L_{\max}$ resulted in a PC (PC1) describing 92.3% of the original variation (eigenvalue = 1.86). $L_{\max}$ loaded negatively (−0.96) while k positively (0.96) on the PC, hence, it described a gradient from fish with large estimated final size and

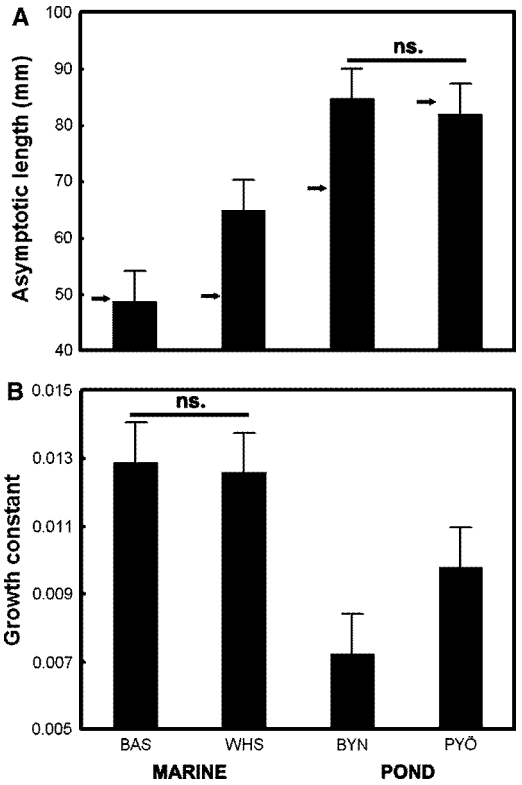


Fig. 2 Population divergence in asymptotic length (L_{max}) and growth constant (k) (=intrinsic growth rate) in nine-spined sticklebacks. Least Squares means +95% Confidence Intervals are shown. Post hoc pairwise comparisons not marked as 'ns.' are significant. *Arrows* in (**a**) denote the maximum size in the given population observed in the wild. *BAS* Baltic Sea, *WHS* White Sea, *BYN* Bynästjärnen (pond), and *PYÖ* Pyöreälampi (pond)

low intrinsic growth rate towards fish with small estimated final size but high intrinsic growth rate. The GLMMs on the PC scores fully supported the analyses ran on L_{max} and k as separate variables (see above). We found a significant habitat effect where marine fish had small estimated final size but high intrinsic growth rate and pond fish the opposite ($F_{1,\ 3.96} = 36.23$; $P = 0.004$; LS mean ± SE: marine = 0.75 ± 0.18; pond = −0.82 ± 0.19). The population effect was nonsignificant (Z = 0.56; $P = 0.58$) while the family effect approached significance (Z = 1.91; $P = 0.06$). The population level GLMM revealed a significant population effect ($F_{3,\ 17.95} = 31.79$; $P < 0.001$). The family effect approached significance (Z = 1.89; $P = 0.06$). Apart from the two marine ($P = 0.18$) and two pond ($P = 0.27$) populations, all other pairs of population differed from each other (all $P < 0.003$) in the expected way (PC1 marine > PC1 pond).

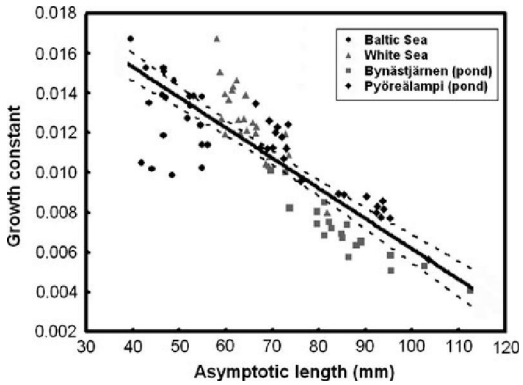


Fig. 3 The relationship between asymptotic length (L_{max}) and growth constant (k) (=intrinsic growth rate) in nine-spined sticklebacks. The *dotted lines* denote 95% Confidence Intervals

Even though L_0 holds no biological information in our case, we report its values for comparative purposes here: means ± SE; Baltic Sea: 5.19 mm ± 0.20; White Sea: 3.38 mm ± 0.15; Bynästjärnen: 5.03 mm ± 0.18; Pyöreälampi: 4.68 mm ± 0.15.

The restricted GLMMs testing for sex effects revealed significant population × sex interaction in both L_{max} (population: $F_{2,\ 12.30} = 140.56$; $P < 0.001$; sex: $F_{1,\ 64.22} = 144.72$; $P < 0.001$; population × sex: $F_{2,\ 63.82} = 26.59$; $P < 0.001$; Fig. 4a) and k (population: $F_{2,\ 14.10} = 14.51$; $P < 0.001$; sex: $F_{1,\ 66.04} = 19.58$; $P < 0.001$; population × sex: $F_{2,\ 66.09} = 5.36$; $P = 0.007$; Fig. 4b). The family effect approached significance for L_{max} (Z = 1.77; $P = 0.07$), and was nonsignificant for k (Z = 1.50; $P = 0.13$). In both variables, the difference between marine and pond fish was more pronounced in females than in males.

In general, marine populations had similar intrinsic growth rates and reached their estimated final lengths faster than pond populations. Pond populations had similar estimated final lengths, larger than marine populations (Fig. 5).

The largest fish from the Baltic Sea ($N = 59$; max standard length = 49.46 mm) and Pyöreälampi ($N = 158$; max standard length = 84.39 mm) did not differ from the L_{max} estimated for the given population (one-sample t-tests: all $P > 0.15$; Fig. 2a). However, the largest fish from the White Sea ($N = 55$; max standard length = 49.99 mm) and Bynästjärnen ($N = 91$; max standard length = 68.30 mm) were smaller than the estimated L_{max} for their respective populations (one-sample t-tests: all $P < 0.001$; Fig. 2a).

Discussion

Our results demonstrate that marine nine-spined stickleback populations have evolved similar intrinsic growth rates—higher than those of pond populations—but variable estimated final sizes. Conversely, pond populations have evolved similar estimated final sizes—larger than marine—but variable intrinsic growth rates. Intrinsic growth rate and estimated final size were negatively correlated, irrespective of population of origin. Growth strategy divergence was more pronounced in females than in males.

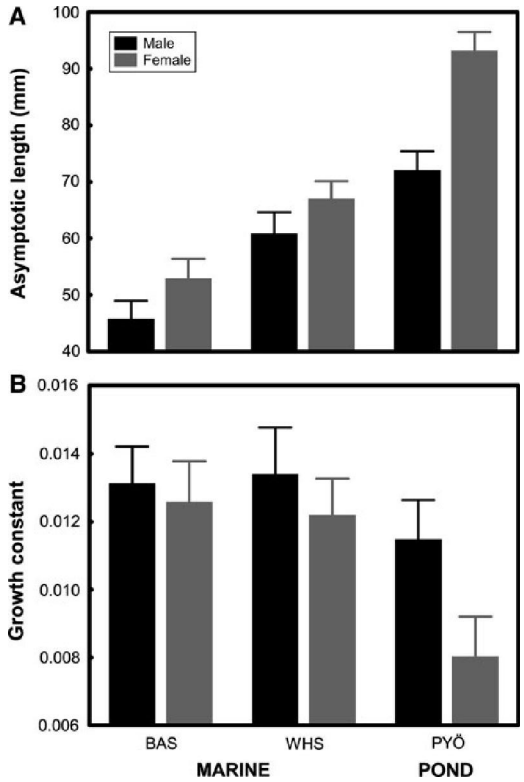


Fig. 4 Population and sex divergence in asymptotic length (L_{max}) and growth constant (k) (=intrinsic growth rate) in nine-spined sticklebacks. Least Squares means +95% Confidence Intervals are shown. *BAS* Baltic Sea, *WHS* White Sea, and *PYÖ* Pyöreälampi (pond)

We have previously shown that in ponds where predation is negligible, nine-spined sticklebacks evolved to antisocial, aggressive and bold giants that initiate feeding quickly, and have higher than expected (based on the allometric body size—metabolic rate relationship) metabolic rate (Herczeg et al. 2009a, b, c, 2010a; Waser et al. 2010). While we found that pond fish had higher year-to-year adult growth than lake or coastal marine fish in nature (Herczeg et al. 2009a; note that fish have indeterminate growth), no information was available from growth in the first year, when most of the growth takes place in the species (Jones and Hynes 1950; Bănărescu and Paepke 2001). We also knew that giant females have 2–3 times higher fecundity than normal-sized females (Herczeg et al. 2010a). We hypothesised that for maximizing lifetime reproductive success, the priority in pond environments with negligible predation is to reach large final body size, while in coastal marine environments with a diverse fish community including several predator species the priority is to reach final body size quickly. Our data are compatible with the hypothesis. In high predation risk environments, reaching a size needed for successful reproduction

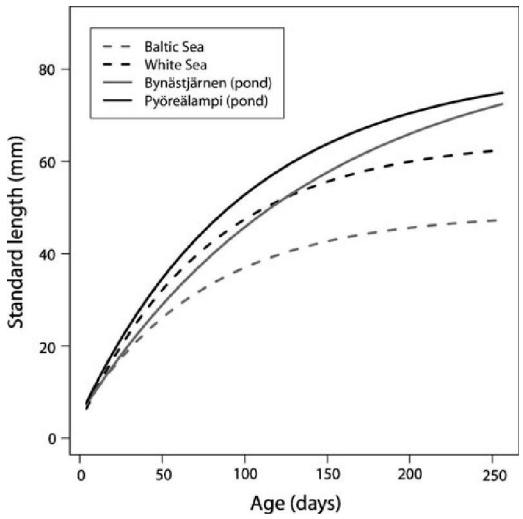


Fig. 5 Mean von Bertalanffy growth curves for the four nine-spined stickleback populations

quickly and allocating mainly to survival/maintenance/reproduction afterwards is likely to maximize fitness. In contrast, in low predation risk environments with longer life expectancy and high intraspecific competition, simply growing to a maximum size—given the various physiological and phylogenetic constraints—is likely to maximize fitness. We observed a strong negative correlation between estimated final body size and growth rate, suggesting a trade-off between these two traits. This is not unexpected based on the energetic trade-offs between growth and reproduction/maintenance (e.g. Roff 1992; Stearns 1992).

In a previous study based on 11 nine-spined stickleback populations, we found female biased sexual size dimorphism (SSD) in the wild, the level of SSD increasing with mean size in the population (Herczeg et al. 2010a). This strong inverse Rensch rule (e.g. Rensch 1950, 1959; Fairbairn 1997) suggests that females are driving body size evolution in this species, while male body size follows females due to correlative selection. This can be understood in the light of the 2–3 times higher fecundity of giant pond as compared to normal-sized marine females (Herczeg et al. 2010a). Our current results on sexual dimorphism in growth strategy gives further support for the role of females: habitat-dependent divergence in both estimated final body size and intrinsic growth rate was more pronounced in females than in males.

Growth is a highly plastic trait responding to many environmental factors (for review see Dmitriew 2011). For instance, low nutrition level or physiologically suboptimal temperature can result in lowered growth rates (Arendt 1997; Dmitriew 2011). However, in our experiment we fed the fish *ad libitum*, kept them individually to avoid the possible adverse social effects (Herczeg et al. 2009b), and set the temperature close to the upper margin of the reported preference range of the species (Lachance et al. 1987). Because of the design, our fish were free from ecological constraints stemming from predation, inter- or intraspecific competition, parasitism or reproduction. As such, they were expected to express

their maximal undisturbed growth performance. Population comparisons of growth rates along environmental gradients show that growth rates usually increase towards higher latitudes, apparently to compensate for the short length of the growing season and/or to reach the size needed for surviving winter (Conover and Present 1990; Blanckenhorn and Demont 2004; Lindgren and Laurila 2005, 2009). However, our samples should not be biased in this respect, as marine populations were from the highest and lowest latitudes sampled, whereas the pond populations came from intermediate latitudes. We note that our high predation populations were from coastal marine environments while the low predation populations from freshwater ponds, hence, the reported patterns might result from salinity differences and not differences in the predatory regime. However, besides the fact that our coastal marine sites already represented low salinity habitats, several other freshwater habitats like large lakes or rivers contain sticklebacks with size and year-to-year adult growth patterns similar to the marine populations (Herczeg et al. 2009a; see also Bănărescu and Paepke 2001), making it unlikely that salinity is the selective agent driving the divergence in growth patterns. With only two replicate populations for each habitat type, the generalization is problematical, but the facts that (i) our within-habitat type replicates were separated by more than 500 km, and (ii) by analysing 13 neutral markers Shikano et al. (2010) found these populations genetically isolated makes them truly independent. Further, analyses of mtDNA sequences revealed that the four populations belong to the same lineage with no sign of difference in time of divergence (Shikano et al. 2010). Hence, the observed patterns are suggested to be general and present in different populations.

We also found that in two cases (one marine and one pond population), the estimated final body size was well beyond the size of the largest fish we measured in the wild. Getting a reliable estimate of asymptotic size in a wild population is notoriously difficult in taxa with indeterminate growth. Using mean size can be misleading, especially in species that live through several growth seasons. Therefore, different approaches, like the use of maximum values (Stamps and Andrews 1992), 90 percent upper percentiles (e.g. Kratochvíl and Frynta 2002) or mean of the five largest individuals (Forsman 1991), are recommended. However, environmentally induced plasticity often masks genetic patterns of growth (e.g. Conover and Present 1990; Laugen et al. 2003), which might be the case with nine-spined sticklebacks. The fact that the fish from the parental White Sea generation were still much smaller than their offspring at the end of the experiment (wild caught fish from Bynästjärnen were not available) suggests that there is a critical time window for intense growth in the first year of growth for which adults cannot compensate later on (personal observation). Of course, there is a chance that our samples from the wild are not representative. However, we have been sampling in Bynästjärnen for several years—and in different seasons—and have handled a large number of unmeasured fish. We have never observed wild sticklebacks as large as those seen at the end of our common garden experiment. Even the maximum body size in the wild can only be treated as a rough proxy for the genetically determined body size in a population.

While predation represents the most striking difference in the biotic environment of coastal marine versus pond habitats, there are other factors that have to be taken into consideration when interpreting the results. Interspecific competition, by constraining individuals' possibilities for utilizing critical resources, can counteract selection favouring large body size (Wilson 1975; Lomolino 1985; Simberloff et al. 2000). Pond sticklebacks are facing negligible interspecific competition as they are the only fish species present, and thus have the possibility to break out from their 'size niche'. Further, in ponds, under negligible predation and interspecific competition, success in intraspecific competition/

resource utilization might be the key to increase fitness. Indeed, in earlier studies we found that pond sticklebacks are more aggressive, and faced with a higher cost from group living as compared to marine conspecifics even in the absence of ecological constraints (Gonda et al. 2009; Herczeg et al. 2009b, c). These results suggest high intraspecific competition in ponds, where stickleback density is much higher than in marine (or large lake) sites (Gabor Herczeg, personal observation). Taken together, negligible predation (and interspecific competition) might have resulted in the divergent growth strategy in ponds by making success in intraspecific competition (=large body size) the most important factor determining fitness.

Finally, it may be instructive to note that in the closely related three-spined stickleback, the opposite pattern of body size differentiation to our results has been reported: gigantism in three-spined sticklebacks occurred in freshwater habitats with sympatric predatory fish (Moodie 1972a, b; Moodie and Reimchen 1976). However, in the case of three-spined sticklebacks the dorsal and pelvic spines together with the supporting bony lateral plates provide effective means of antipredator defence (e.g. Hoogland et al. 1957; Reimchen 1983), while the spines of nine-spined sticklebacks are far less effective against predators (Hoogland et al. 1957). Hence, large body size can serve three-spined sticklebacks to escape from size-limited predators, and as a consequence, presence of size-limited predators can select for gigantism in this species (e.g. Moodie 1972a, b; Moodie and Reimchen 1976; Reimchen 1988, 1991). However, in our case, marine nine-spined sticklebacks are faced with size-unlimited predation, and the larger body size of pond nine-spine sticklebacks occurs parallel to the reduction or even complete loss of the pelvic apparatus in the absence of predatory fish (Herczeg et al. 2010b).

In summary, we found different growth strategies between coastal marine and pond nine-spined sticklebacks. Marine fish grew to small sizes but reached them quickly, while pond fish grew to large sizes but reached them slowly. This reflects the negative correlation between intrinsic growth rate and estimated final body size. Further experiments are required to identify the selective factor(s) behind the reported growth strategy divergence unequivocally. For instance, establishing both the heritabilities of the growth parameters and the strength of the selection on them imposed by predation, competition and salinity, or monitoring evolution taking place in semi-natural enclosures with manipulated predation, competition and salinity might provide next steps towards understanding relative importance of selective factors behind growth rate divergence.

Acknowledgments We thank Victor Berger, Göran Englund, Tuomas Leinonen, Daniel Lussetti, and Pirkko Siikamäki for their help in organising and executing field sampling. Special thanks to the Oulanka Research Station (University of Oulu) and White Sea Biological Station (Russian Academy of Science) for sharing their facilities and expertise. We are highly indebted to Caitlin Dmitriew and two anonymous reviewers for their comments leading to improvements of our manuscript and Jacquelin DeFaveri for correcting the English. The authors received financial support from the Academy of Finland (GH, AK, JM) and Center of Internal Mobility (CIMO) (AG). The experiments were conducted under the licence of the Finnish National Animal Experiment Board (ELLA, # STH379A).

References

Abrams PA, Rowe L (1996) The effects of predation on the age and size of maturity of prey. Evolution 50:1052–1061

Andersson M (1994) Sexual selection. Princeton University Press, Princeton

Arendt JD (1997) Adaptive intrinsic growth rates: an integration accross taxa. Q Rev Biol 72:149–177

Arendt JD, Reznick DN (2005) Evolution of juvenile growth rates in female guppies (*Poecilia reticulata*): predator regime or resource level? Proc R Soc B 272:333–337
Atchley WR (1984) Ontogeny, timing of development, and genetic variance-covariance structure. Am Nat 123:519–540
Bănărescu PM, Paepke H-J (2001) The freshwater fishes of Europe, vol 5/III. AULA, Wiebelsheim
Barrett RDH, Rogers SM, Schluter D (2008) Natural selection on a major armor gene in three spine stickleback. Science 322:255–257
Bell MA, Ortí G, Walker JA, Koenings JP (1993) Evolution of pelvic reduction in three spine stickleback fish—a test of competing hypotheses. Evolution 47:906–914
Berrigan D, Charnov EL (1994) Reaction norms for age and size at maturity in response to temperature: a puzzle for life historians. Oikos 70:474–478
Biro PA, Abrahams MV, Post JR, Parkinson EA (2004) Predators select against high growth rates and risk-taking behaviour in domestic trout populations. Proc R Soc Lond B 271:2233–2237
Biro PA, Abrahams MV, Post JR, Parkinson EA (2006) Behavioural trade-offs between growth and mortality explain evolution of submaximal growth rates. J Anim Ecol 75:1165–1171
Blanckenhorn WU (2000) The evolution of body size: what keeps organisms small? Q Rev Biol 75:385–407
Blanckenhorn WU, Demont M (2004) Bergmann and converse Bergmann latitudinal clines in arthropods: two ends of a continuum? Integr Comp Biol 44:413–424
Calow P (1982) Homeostasis and fitness. Am Nat 120:416–419
Conover DO, Present TMC (1990) Countergradient variation in growth rate: compensation for length of the growing season among Atlantic silversides from different latitudes. Oecologia 83:316–324
Day T, Rowe L (2002) Developmental thresholds and the evolution of reaction norms for age and size at maturity. Am Nat 159:338–350
Dmitriew CM (2011) The evolution of growth trajectories: what limits growth rate? Biol Rev 86:97–116
Fairbairn DJ (1997) Allometry for sexual size dimorphism: patterns and process in the coevolution of body size in males and females. Ann Rev Ecol Syst 28:659–687
Forsman A (1991) Variation in sexual size dimorphism and maximum body size among adder populations: effects of prey size. J Anim Ecol 60:253–267
Giles N (1983) The possible role of environmental calcium levels during the evolution of phenotypic diversity in Outer-Hebridean populations of the three-spined stickleback, *Gasterosteus aculeatus*. J Zool 199:535–544
Gonda A, Herczeg G, Merilä J (2009) Habitat-dependent and–independent plastic responses to social environment in the nine-spined stickleback (Pungitius pungitius) brain. Proc Roy Soc B 276:2085–2092
Green BS (2008) Maternal effects in fish populations. Adv Mar Biol 54:1–105
Heino M, Kaitala V (1999) Evolution of resource allocation between growth and reproduction in animals with indeterminate growth. J Evol Biol 12:423–429
Herczeg G, Gonda A, Merilä J (2009a) Evolution of gigantism in nine-spined sticklebacks. Evolution 63:3190–3200
Herczeg G, Gonda A, Merilä J (2009b) The social cost of shoaling covaries with predation risk in nine-spined stickleback (*Pungitius pungitius*) populations. Anim Behav 77:575–580
Herczeg G, Gonda A, Merilä J (2009c) Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). J Evol Biol 22:544–552
Herczeg G, Gonda A, Merilä J (2010a) Rensch's rule inverted—female-driven gigantism in nine-spined stickleback (Pungitius pungitius). J Anim Ecol 79:581–588
Herczeg G, Turtiainen M, Merilä J (2010b) Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. Biol J Linn Soc 101:413–416
Hoogland R, Morris D, Tinbergen N (1957) The spines of sticklebacks (*Gasterosteus* and *Pygosteus*) as means of defence against predators (*Perca* and *Esox*). Behaviour 10:205–236
Jones JW, Hynes HBN (1950) The age and growth of *Gasterosteus aculeatus*, *Pygosteus pungitius* and *Spinachia vulgaris* as shown by their otoliths. J Anim Ecol 19:59–73
Kahilainen K, Malinen T, Tuomaala A, Lehtonen H (2004) Diel and seasonal habitat and food segregation of three sympatric *Coregonus lavaretus* forms in a subarctic lake. J Fish Biol 64:418–434
Katsanevakis S (2006) Modelling fish growth: model selection, multi-model inference and model selection uncertainty. Fish Res 81:229–235
Kratochvíl L, Frynta D (2002) Body size, male combat and the evolution of sexual dimorphism in *Eublepharid geckos* (Squamata: Eublepharidae). Biol J Linn Soc 76:303–314
Kuparinen A, Cano Arias JM, Loehr J, Herczeg G, Gonda A, Merilä J (2011) Fish age at maturation is influenced by temperature independently of growth. Oecologia. doi: 10.1007/s00442-011-1989-x

Lachance S, Magnan P, FitzGerald GJ (1987) Temperature preferences of three sympatric sticklebacks (Gasterosteidae). Can J Zool 65:1573–1576
Laugen AT, Laurila A, Räsänen K, Merilä J (2003) Latitudinal countergradient variation in the common frog (*Rana temporaria*) development rates—evidence for local adaptation. J Evol Biol 16:996–1005
Lima SL, Dill LM (1990) Behavioural decisions made under the risk of predation- a review and prospectus. Can J Zool 68:619–640
Lindgren B, Laurila A (2005) Proximate causes of adaptive growth rates: growth efficiency variation among latitudinal populations of *Rana temporaria*. J Evol Biol 18:820–828
Lindgren B, Laurila A (2009) Physiological variation along a geographical gradient: is growth rate correlated with routine metabolic rate in *Rana temporaria* tadpoles? Biol J Linn Soc 98:217–224
Lomolino MV (1985) Body size of mammals on islands: the island rule re-examined. Am Nat 125:310–316
Marchinko KB, Schluter D (2007) Parallel evolution by correlated response: lateral plate reduction in three spine stickleback. Evolution 61:1084–1090
Moodie GEE (1972a) Predation, natural selection and adaptation in an unusual three spine stickleback. Heredity 28:155–167
Moodie GEE (1972b) Morphology, life history and ecology of an unusual stickleback (*Gasterosteus aculeatus*) in the Queen Charlotte Islands. Can J Zool 50:721–738
Moodie GEE, Reimchen TE (1976) Phenetic variation and habitat differences in *Gasterosteus* populations of the Queen Charlotte Islands. Syst Zool 25:49–61
Östlund-Nilsson S, Mayer I, Huntingford FA (2007) Biology of the three-spined stickleback. CRC Press, Boca Raton
Peters RH (1983) The ecological implications of body size. Cambridge University Press, Cambridge
R Development Core Team (2009) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0. URL http://www.R-project.org
Reimchen TE (1983) Structural relationships between spines and lateral plates in threespine stickleback (*Gasterosteus aculeatus*). Evolution 37:931–946
Reimchen TE (1988) Inefficient predators and prey injuries in a population of giant stickleback. Can J Zool 66:2036–2044
Reimchen TE (1991) Trout foraging failure and the evolution of body size in stickleback. Copeia 1991:1098–1104
Rensch B (1950) Die Abhängigkeit der relativen Sexualdifferenz von der Körpergrösse. Bonn Zool Beitr 1:58–69
Rensch B (1959) Evolution above species level. Columbia University Press, New York
Roff DA (1992) The evolution of life histories. Chapman & Hall, New York
Rohlf FJ (2006) TPS software series. Distributed by the author. Department of ecology and evolution, State University of New York, Stony Brook
Shikano T, Shimada Y, Herczeg G, Merilä J (2010) History versus habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. Mol Ecol 19:1147–1161
Shine R (1989) Ecological causes for the evolution of sexual size dimorphism: a review of the evidence. Q Rev Biol 64:419–461
Sibly R, Calow P (1986) Why breeding earlier is always worthwhile. J Theor Biol 124:311–319
Simberloff D, Dayan T, Jones C, Ogura G (2000) Character displacement and release in the small Indian mongoose, *Herpestes javanicus*. Ecology 81:2086–2099
Stamps JA, Andrews RM (1992) Estimating asymptotic size using the largest individual per sample. Oecologia 92:503–512
Stearns SC (1992) The evolution of life histories. Oxford University Press, Oxford
Urban MC (2008) Salamander evolution across a latitudinal cline in gape-limited predation risk. Oikos 117:1037–1049
von Bertalanffy L (1938) A quantitative theory of organic growth (inquiries on growth laws II). Human Biol 10:181–213
Waser W, Sahoo TP, Herczeg G, Merilä J, Nikinmaa M (2010) Physiological differentiation among nine-spined stickleback populations: effects of copper exposure. Aquat Toxicol 98:188–195
Werner EE, Gilliam JF (1984) The ontogenetic niche and species interactions in size structured populations. Ann Rev Ecol Syst 15:393–425
Wilson DS (1975) The adequacy of body size as a niche difference. Am Nat 109:769–784

JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12409

Evidence for sex-specific selection in brain: a case study of the nine-spined stickleback

G. HERCZEG*†, K. VÄLIMÄKI†‡, A. GONDA† & J. MERILÄ†

*Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Budapest, Hungary
†Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, Helsinki, Finland
‡Monitoring Team, Finnish Museum of Natural History, University of Helsinki, Helsinki, Finland

Keywords:

adaptation;
brain;
food;
plasticity;
predation;
Pungitius;
sexual dimorphism;
stickleback.

Abstract

Theory predicts that the sex making greater investments into reproductive behaviours demands higher cognitive ability, and as a consequence, larger brains or brain parts. Further, the resulting sexual dimorphism can differ between populations adapted to different environments, or among individuals developing under different environmental conditions. In the nine-spine stickleback (*Pungitius pungitius*), males perform nest building, courtship, territory defence and parental care, whereas females perform mate choice and produce eggs. Also, predation-adapted marine and competition-adapted pond populations have diverged in a series of ecologically relevant traits, including the level of phenotypic plasticity. Here, we studied sexual dimorphism in brain size and architecture in nine-spined stickleback from marine and pond populations reared in a factorial experiment with predation and food treatments in a common garden experiment. Males had relatively larger brains, larger *telencephala*, *cerebella* and *hypothalami* (6 16% divergence) than females, irrespective of habitat. Females tended to have larger *bulbi olfactorii* than males (13%) in the high food treatment, whereas no such difference was found in the low food treatment. The strong sexual dimorphism in brain architecture implies that the different reproductive allocation strategies (behaviour *vs.* egg production) select for different investments into the costly brains between males and females. The lack of habitat dependence in brain sexual dimorphism suggests that the sex-specific selection forces on brains differ only negligibly between habitats. Although significance of the observed sex-specific brain plasticity in the size of *bulbus olfactorius* remains unclear, it demonstrates the potential for sex-specific neural plasticity.

Introduction

Variation in brain size and architecture in the wild have always attracted great scientific interest, and thanks to this continued attention, considerable variation has been uncovered in diverse taxa (e.g. Harvey *et al.*, 1980; Kotrschal *et al.*, 1998; Day *et al.*, 2005; Striedter, 2005). For instance, interspecific comparative studies have revealed correlations between brain size/architecture and environmental factors (Pollen *et al.*, 2007), life history (Gonzalez-Voyer *et al.*, 2009a; Barton & Capellini, 2012), behaviour (Ratcliffe *et al.*, 2006; Aviles & Garamszegi, 2007) and morphology (Aiello & Wheeler, 1995; Pitnick *et al.*, 2006; Gonzalez-Voyer *et al.*, 2009b). Likewise, recent studies employing intraspecific population comparisons have uncovered habitat-dependent variation in brain size and architecture (Gonda *et al.*, 2009a, 2011; Kolm *et al.*, 2009; Roth & Pravosudov, 2009; Møller, 2010; Park & Bell, 2010; Roth *et al.*, 2012). Besides the presumably genetically based variation, several forms of phenotypic plasticity (i.e. the capacity of producing different phenotypes from a single genotype, West-Eberhard, 2003) have also been proven at different neuroanatomical levels in diverse taxa (reviewed in Van Praag *et al.*, 2000; Mohammed *et al.*, 2002). However, only few studies (but see: Nottebohm & Arnold, 1976; Kotrschal *et al.*, 2012a) have focussed on sexual dimorphism in brain size and architecture.

According to Jacobs (1996), cognitive ability and, thus, the related neural capacity should be more developed in the sex with higher behavioural investments into reproduction. Interspecific comparative studies support this view. For instance, bower complexity correlates positively with brain size in bowerbirds (Madden, 2001), uniparental care occurs in cichlids with larger brains (Gonzalez-Voyer *et al.*, 2009a), and the intensity of sexual selection correlates with brain size (Fitzpatrick *et al.*, 2012). In passerines with high rate of extra-pair paternity females, while in passerines with low rate of extra-pair paternity males have larger brains (Garamszegi *et al.*, 2005a). Further, the degree of sexual dimorphism in brain is higher in passerines where sexual dimorphism in song complexity is also high (Garamszegi *et al.*, 2005b). Nevertheless, intraspecific evidence for sexual dimorphism in brain is still scarce, especially when the data are properly analysed and interpreted (Kotrschal *et al.*, 2012a). It has been shown that in two distinct three-spined stickleback (*Gasterosteus aculeatus*) morphs, males have ca. 23% heavier brains than females (Kotrschal *et al.*, 2012a), possibly as a result of the different sexual roles during reproduction.

Further, the interplay between different sources of variation in brain size and architecture (e.g. local adaptation × sexual dimorphism; sexual dimorphism × phenotypic plasticity) has rarely been addressed. For instance, African cichlids (*Pseudocrenilabrus multicolor victoriae*) with high dispersal potential have more plastic brains than cichlids with low dispersal potential (Crispo & Chapman, 2010). Nine-spined sticklebacks (*Pungitius pungitius*) also show habitat-dependent population divergence in brain plasticity induced by social environment (Gonda *et al.*, 2009b) and perceived predation risk (Gonda *et al.*, 2012). Sex-specific phenotypic plasticity in the brain has been demonstrated only recently: Kotrschal *et al.* (2012b) reported that manipulation of the social environment induced sex-specific responses in the guppy, *Poecilia reticulata*. We are not aware of any study reporting habitat-dependent sexual dimorphism in brain.

The nine-spined stickleback system in Fennoscandia provides an excellent model to study sexual dimorphism and interacting effects of different factors on brain development. The species is widely distributed and occupies different habitats with varying selection regimes (e.g. Wootton, 1976; Östlund-Nilsson *et al.*, 2007; Merilä, 2013). They show habitat-dependent population divergence (*viz* predation-adapted marine *vs.* competition-adapted pond populations) in life history and behaviour (Herczeg *et al.*, 2009a,b,c, 2012; Ab Ghani *et al.*, 2012, 2013; Aikio *et al.*, 2013), including brain size and architecture (Gonda *et al.*, 2009a, 2011). Furthermore, habitat-dependent phenotypic plasticity (induced by perceived predation risk and available energy) in life-history and behavioural traits (Herczeg & Välimäki, 2011; Välimäki & Herczeg, 2012) as well as in brain architecture (Gonda *et al.*, 2009b, 2012) has been demonstrated. In sticklebacks, males perform various reproduction-related behaviours (nest building, territoriality, female-attraction display, paternal care) possibly selecting for large brain, whereas females do not (e.g. Bell & Foster, 1994; Östlund-Nilsson *et al.*, 2007). However, females do perform mate choice and thus should be able to assess and memorize different males (including male ornaments, male courtship displays and male nests; Milinski & Bakker, 1992), which might also select for high cognitive ability demanding larger brains. On the other hand, females invest a lot into reproduction, in three-spined sticklebacks the weight of gonads can account for 40% of the total body weight (Bell & Foster, 1994), and thus, a trade-off between reproductive investment and brain development is feasible (Aiello & Wheeler, 1995).

The main aim of the current study was to test for importance of sex as a source of variation in brain size and architecture in the nine-spined stickleback. Besides sex effects, we also investigated how the interplay between sex and local adaptation (*viz.* habitat of origin) and between sex and phenotypic plasticity (*viz.* environment during development) affects brain morphology. We predicted more developed (i.e. larger) brains in male than in female nine-spined sticklebacks, with lower degree of dimorphism in pond populations where sticklebacks invest much energy into somatic growth (Herczeg *et al.*, 2009a, 2012; Aikio *et al.*, 2013) or when developing under suboptimal conditions. To test these ideas, we conducted a common garden experiment using two marine (predation-adapted) and two genetically and geologically distant pond populations (competition-adapted). We collected adult fish from the wild and bred them in laboratory. The F1 generation of offspring was subjected to a full factorial common garden experiment with two levels of predation (presence *vs.* absence of perceived predation risk) and food (high *vs.* low) treatments until they approached mature size.

Materials and methods

Sampling and experimental design

The experiment is the same as described in detail in Herczeg and Välimäki (2011) and Välimäki and Herczeg (2012). The brain data are the same as the one used by Gonda *et al.* (2012), where the focus was on habitat effects and the habitat × treatment interactions. Gonda *et al.* (2012) did not present or discuss the sex effects, but simply standardized away sex differences by including sex as a factor into the models.

Correspondence: Gábor Herczeg, Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/C, 1117, Hungary.
Tel.: +36 1 372 2500/8760; fax: +36 1 381 2194;
e-mail: gaborherczeg@caesar.elte.hu

© 2014 THE AUTHORS. J. EVOL. BIOL. 27 (2014) 1604–1612
JOURNAL OF EVOLUTIONARY BIOLOGY © 2014 EUROPEAN SOCIETY FOR EVOLUTIONARY BIOLOGY

Briefly, we collected adult nine-spined sticklebacks from two isolated ponds (Abbortjärn, Sweden, 64°29′N, 19°26′E; Pyöreälampi, Finland, 66°15′N, 29°26′E) and two Baltic Sea marine sites (Nyköping, Sweden, 58°39′N; 17°06′E; Helsinki, Finland, 60°13′N, 25°11′E) with seine nets and minnow traps at the beginning of the reproductive season of 2009. The ponds are small (<5 ha surface area), structurally simple isolated water bodies where nine-spined stickleback is the only fish species (apart from introduced whitefish *Coregonus lavaretus* in Pyöreälampi, which can be competitors, but not predators of sticklebacks, Kahilainen *et al.*, 2004), whereas the marine sites are characterized with a more complex biotic and abiotic environment, including numerous predators and competitors.

Adult fish were transported to the aquaculture facilities of the University of Helsinki, where *in vitro* fertilizations were performed during July and August 2009. Altogether 29 full-sib families (Abbortjärn = 6; Pyöreälampi = 7; Nyköping = 8; Helsinki = 8) were produced. Freshly hatched fry were placed individually into 1.4-L containers of four zebrafish racks (Allentown Zebrafish Rack Systems, Allentown Inc., San Diego, CA, USA, hereafter 'rack'). Fish were divided randomly and evenly to the four treatments (see below), and an equal population/family/treatment representation in each rack was aimed for. Water temperature was set to 12 °C and the photoperiod to 14:10 h light:dark. Feeding started with live brine shrimp (*Artemia* sp.) nauplii, followed by feeding with frozen bloodworms (Chironomidae sp.). All rearing was carried out in freshwater.

A full factorial design with treatments (*viz.* predation risk and food level), each with two levels, was applied. For the predation risk treatment (presence/absence of olfactory cues from a common fish predator), we connected one 150-L plastic tank to every racks' water flow. Two 10- to 15-cm-long perch (*Perca fluviatilis*) were placed into two of the external tanks providing the olfactory stimuli and the other two tanks were left as controls. Perch were fed with frozen bloodworms; hence, olfactory cues from the predator alone – not in combination with alarm cues from the attacked and eaten stickleback – were the stimuli. Perch is a common nine-spined stickleback predator (Koli *et al.*, 1988) that is abundant in the Baltic Sea (Ådjers *et al.*, 2006) and probably also the most common predator in the Fennoscandian freshwaters (Koli, 1990). Fish within rack/predation treatment/family were randomly and evenly assigned to the two food treatments. The high food treatment meant two *ad libitum* feedings per day, whereas the low food treatment meant one *ad libitum* feeding in every second day.

Thirty-four-week-old fish were overanaesthetized with tricaine methanesulfonate (MS-222) and their brains were dissected. At this age, fish were reaching adult size, but the artificial environment (10 h darkness per day) did not facilitate fish actually turning into reproductive condition. Dissected brains were fixed in 4% formalin–0.1 M phosphate-buffered saline solution. Fixed brains were photographed from standard angles (viz. dorsal, lateral and ventral) with a digital camera (Nikon D60, Nikon Corporation, Chiyoda, Tokio, Japan) on a tripod with a millimetre scale placed in every photograph. Three-dimensional linear measures were taken from the photographs using tps.Dig 2.15 (Rohlf, 2006). Volume of the whole brain and that of the *bulbus olfactorius*, *telencephalon*, *tectum opticum*, *cerebellum* and *hypothalamus* were estimated applying the ellipsoid model on three-dimensional linear measures (Huber *et al.*, 1997; Pollen *et al.*, 2007) as explained in Gonda *et al.* (2009a,b, 2011, 2012). Sex was identified by gonadal inspection. Fish were also photographed before dissection, and their standard length (from the tip of the nose to the end of the tail base) was measured from the digital photographs using tps.Dig 2.15. Body weight was measured to the nearest 0.1 g with a digital balance.

Statistical analyses

Altogether, 186 brains could be included in the analyses. Therefore, we lacked statistical power to properly estimate family effects and thus simply assumed that the sample of families is representative for the populations (Gonda *et al.*, 2012). All variables were $\log_{10}$-transformed prior analyses. Variation in brain size and size of the different brain parts was analysed with general linear mixed models (GLMMs).

To test for possible differences in (log) body size–(log) brain size allometry between sexes, we ran GLMMs with brain size and size of the different brain parts as dependent variables, sex as a fixed factor, standard length or body weight as a covariate and population within habitat type as a random factor, including the fixed factor × covariate interactions. Because we found that the sex × standard length or sex × body weight interactions were always far from significant (see Results), we assumed that the brain–body size relationship (allometry) is similar in both sexes, and thus, we did not include factor covariate interactions in the main models (see below).

Our main GLMM models included habitat, sex, predation risk treatment and food treatment as fixed factors and population within habitat type as a random factor. With respect to the main factors, we had on average more than 10 individuals in one group (habitat × sex × predation × food: Mean = 11.6, Standard Deviation = 3.8, Minimum = 6, Maximum = 20). However, to avoid overparameterization, we included only the two-way interactions between the fixed factors (three-way interactions did not add any extra significant sex-related effects). For total brain size, body weight was added as covariate, whereas for the brain parts, total brain volume was used as a covariate. We note that these analyses differ from our previous ones (Gonda *et al.*, 2012) because now we only included one covariate per model to avoid multicollinearity. However, this change had only minor effects on the results. Post hoc pairwise comparisons were made with Tukey–Kramer tests. As these GLMMs were not independent, we applied false discovery rate correction (Benjamini & Hochberg, 1995). Statistical analyses were carried out using the SAS 9.2 (SAS institute Inc., Cary, NC, USA) software.

Results

There was no sign of sex-dependent body size allometry in any of the studied brain traits (sex × standard length: all $P > 0.34$; sex × body weight: all $P > 0.32$; the body weight–brain/brain part relationships are shown in Fig. S1). Sexual dimorphism in total brain volume relative to body weight was significant ($F_{1,172} = 137.92$, $P < 0.0001$), as were in *telencephalon* ($F_{1,172} = 54.19$, $P < 0.0001$), *cerebellum* ($F_{1,172} = 14.76$, $P = 0.0002$) and *hypothalamus* ($F_{1,172} = 17.90$, $P < 0.0001$) volume relative to total brain volume too. In all cases, males had larger brains/brain parts than females relative to their size (Fig. 1).

Only one sex-related interaction approached significance: relative size of *bulbus olfactorius* differed between the sexes depending on the food treatment (sex × food treatment: $F_{1,172} = 5.75$, $P = 0.018$; Fig. 2). However, this effect was not significant after the false discovery rate control, hence, it should be interpreted accordingly. According to Tukey–Kramer post hoc tests, males differed from females (males < females) in the high ($P = 0.0064$), but not in the low food treatment ($P = 0.99$). All other effects including sex were nonsignificant (Table 1). The population effects were always nonsignificant (Table 1). We provide the raw means and standard deviations for every variable by population and sex in Table S1.

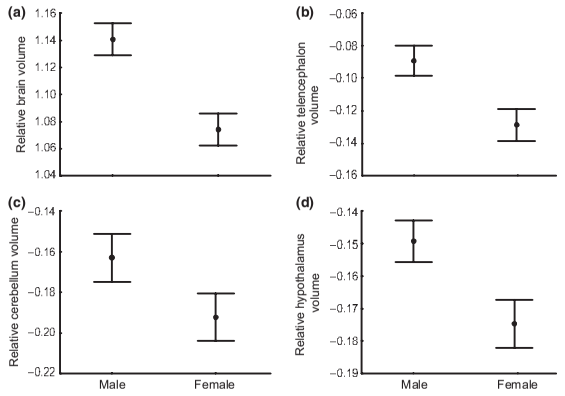


Fig. 1 Sexual size dimorphism in (a) total brain volume, (b) telencephalon volume, (c) cerebellum and (d) hypothalamus volume in nine-spined sticklebacks. Least squares means (± Standard Errors) adjusted for effects of fish weight (a) or for total brain volume (b, c, d) are shown.

Discussion

The main findings of this study were twofold. First, we found strong sexual brain size dimorphism: males had relatively larger brains, *telencephala*, *cerebella* and *hypothalami* than females. Second, we found a trend for an interaction (P = 0.018, marginally significant after a false discovery rate correction) between sex and the food treatment on *bulbus olfactorius* size, sexual dimorphism (females > males) being only evident in the high food treatment. However, contrary to our expectations, neither habitat nor predation treatment affected the degree of the observed sexual dimorphism in brain size and architecture.

Brain tissue is thought to be expensive to develop and maintain (Aiello & Wheeler, 1995; see also Navaterre *et al.*, 2011; Allen & Kay, 2012; Warren & Iglesias, 2012; Kotrschal *et al.*, 2013), and thus, unnecessarily large brains or brain parts are expected to be maladaptive. Therefore, the relative sizes of brain and any given brain parts should be good proxies of their importance in the given context – an idea that has received support in both evolutionary (de Winter & Oxnard, 2001; Gonzalez-Voyer & Kolm, 2010) and ontogenetic (Kihslinger & Nevitt, 2006; Kihslinger *et al.*, 2006; Lisney *et al.*, 2007) studies. Sexual dimorphism in brain anatomy has been documented (e.g. Nottebohm & Arnold, 1976; de Groof *et al.*, 2009; Kotrschal *et al.*, 2012a), but the evolutionary drivers of this divergence have rarely been identified. Jacobs (1996) argued that increased cognitive demands related to reproductive behaviour

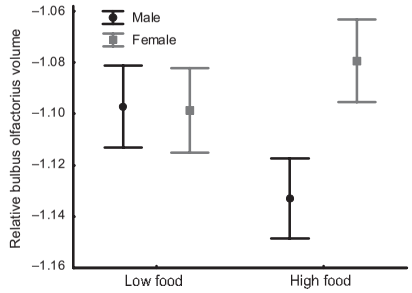


Fig. 2 The effects of sex and food treatment on *bulbus olfactorius* volume. Least squares means (± Standard Errors) adjusted for total brain volume are shown.

might select for increased cognitive ability, and as a consequence, increased neural capacity as reflected in brain size. Therefore, the sex that invests more into reproductive behaviours is expected to be the one with enhanced neural capacity (Jacobs, 1996). In sticklebacks, males build nests, perform various behavioural displays to attract females, defend territories and even provide care for their offspring, whereas females perform mate choice and also provide the costly eggs (e.g. Bell & Foster, 1994; Östlund-Nilsson *et al.*, 2007). Hence, one would expect male sticklebacks to develop relatively larger brains than females. Indeed, we found that male nine-spined sticklebacks had relatively larger brains (16.44%, all percentage calculations were made based on back-transformed least squares means from the models) than females. This finding is in line with that of Kotrschal *et al.*'s (2012a) who reported extreme (23%) male-biased sexual brain size dimorphism in the closely related three-spined stickleback, and in general, with interspecific patterns such as uniparental care selecting for larger brains in cichlids (Gonzalez-Voyer *et al.*, 2009a) as well as bower complexity correlating positively with brain size in bowerbirds (Madden, 2001).

We further found that males had relatively larger *telencephalon* (9.3%), *cerebellum* (6.95%) and *hypothalamus* (6.15%) than females. *Telencephalon* size in fish correlates with habitat complexity (Huber *et al.*, 1997) and has been found to be larger in polygamous than in monogamous species (Pollen *et al.*, 2007). Furthermore, *telencephalon* is known to be involved in various types of learning (e.g. Laming & McKinley, 1990; Broglio *et al.*, 2003; Portavella *et al.*, 2003). Therefore, increased *telencephalon* size in the territorial polygamous male sticklebacks exhibiting paternal care suggests that males need higher cognitive abilities than females. The level of differentiation we found, 9.3%, could be considered as high. Hence, by inference, cognitive demands presumed to drive sexual differentiation in *telencephalon* size between the sexes can be expected to be substantial. *Cerebellum* has its role in spatial orientation, eye movement and motor coordination (Kotrschal *et al.*, 1998). These traits are all of high importance for male sticklebacks building their complex nests and guarding their swimming offspring; hence, sexual dimorphism in *cerebellum* (6.95%) is easy to interpret. The *hypothalamus* has various functions, including reproductive and feeding behaviour (e.g. White & Fernald, 1993; Kotrschal *et al.*, 1998; Kulczykowska & Sánchez Vázquez, 2010). Hence, the male-biased sexual *hypothalamus* size dimorphism (6.15%) is not straightforward to interpret, but it can be suspected that it might be also related to the sex role differences. The reported sexual divergence is often comparable to what we have found earlier between populations residing to different habitats (Gonda *et al.*, 2009a; Gonda *et al.*, 2011): for instance, in the present experiment, marine fish had 34.15% larger *bulbi olfactorii* than pond fish, whereas the sex difference in the high food treatment amounted to 13.14%. Likewise, while marine fish had 13.82% larger *telencephala* than pond fish (Gonda *et al.*, 2009a,b), the sex difference in the present study amounted to 9.3%. Hence, it appears that sex-specific selection pressures contribute significantly to those related to habitat differences resulting in the observed intraspecific variation.

Table 1 Results of the GLMMs. *F* statistics (with degrees of freedom in parenthesis) and *P* values are shown. In case of population (random factor), *Z* statistics and *P* values (in parentheses) are shown. Bold values indicate significant effects after false discovery rate correction. Note that we focus on sex effects here, habitat effects were discussed elsewhere (Gonda *et al.*, 2012).

Effect	Total brain	Bulbus olfactorius	Telencephalon	Tectum opticum	Cerebellum	Hypothalamus
Sex (S)	**137.92 (1,172) <0.0001**	4.60 (1,172) 0.033	**54.19 (1,172) <0.0001**	0.30 (1,172) 0.58	**14.76 (1,172) 0.0002**	**17.90 (1,172) <0.0001**
Habitat (H)	3.49 (1,2.22) 0.19	24.74 (1,2.2) 0.031	0.58 (1,2.08) 0.52	0.42 (1,2.07) 0.58	0.83 (1,2.11) 0.45	0.56 (1,2.38) 0.52
Predation (P)	0.01 (1,173) 0.92	2.91 (1,173) 0.09	2.20 (1,172) 0.14	0.06 (1,172) 0.81	0.21 (1,173) 0.65	3.35 (1,172) 0.07
Food (F)	0.38 (1,172) 0.54	0.50 (1,173) 0.48	1.11 (1,172) 0.29	0.29 (1,172) 0.59	0.04 (1,172) 0.84	0.01 (1,173) 0.90
S × H	0.28 (1,172) 0.60	0.10 (1,172) 0.76	0.09 (1,172) 0.77	1.01 (1,172) 0.32	0.12 (1,172) 0.73	0.37 (1,172) 0.54
S × P	0.21 (1,172) 0.64	0.91 (1,172) 0.34	0.81 (1,172) 0.37	0.11 (1,172) 0.74	0.03 (1,172) 0.87	**<0.01 (1,172) 0.96**
S × F	0.67 (1,172) 0.42	5.75 (1,172) 0.018	0.04 (1,172) 0.83	0.40 (1,172) 0.53	0.16 (1,172) 0.69	0.20 (1,172) 0.65
H × P	**<0.01 (1,172) 0.95**	3.63 (1,173) 0.06	0.02 (1,172) 0.89	0.05 (1,172) 0.82	0.01 (1,172) 0.93	0.22 (1,173) 0.64
H × F	2.91 (1,172) 0.09	0.03 (1,172) 0.87	0.04 (1,172) 0.84	0.04 (1,172) 0.85	0.05 (1,172) 0.83	0.07 (1,172) 0.79
P × F	2.89 (1,172) 0.09	0.13 (1,173) 0.72	0.30 (1,172) 0.57	2.88 (1,172) 0.09	0.13 (1,172) 0.72	0.09 (1,173) 0.77
Body weight	**424.03 (1,174) <0.0001**	–	–	–	–	–
Total brain	–	**144.20 (1,174) <0.0001**	**506.70 (1,173) <0.0001**	**1340.86 (1,173) <0.0001**	**522.22 81,173) <0.0001**	**282.48 (1,174) <0.0001**
Population	0.93 (0.18)	0.78 (0.22)	0.92 (0.18)	0.92 (0.18)	0.90 (0.18)	0.76 (0.22)

We have shown previously that nine-spined sticklebacks are sexually size dimorphic (Herczeg *et al.*, 2010). Hence, one might consider the sexual dimorphism observed in brain being a direct consequence of sexual dimorphism in size. However, it is easy to reject this hypothesis: (i) the raw data presented in Table S1 show that whereas females are the larger sex, males have larger brains and brain parts already in absolute terms, (ii) our analyses compared brains corrected for size, meaning that the reported sexual dimorphism in brain is size-independent, and finally, (iii) we tested for sex-specific brain size–body size allometry, but found none (see Results). Hence, it is feasible to suggest that different selective forces resulted in the body size sexual dimorphism than in brain sexual dimorphism. We suggested that body size sexual dimorphism is a result of fecundity selection acting on females under negligible predation (hence that habitat dependence of body size sexual dimorphism, Herczeg *et al.*, 2010), whereas we suggest that sexual selection acting on males' behavioural investment during reproduction is responsible for brain sexual dimorphism.

Evolutionary ecologists are often interested in interactions between different factors as drivers of phenotypic divergence among populations or sexes. With regard to sexual size dimorphism, earlier studies have found evidence for sex × habitat interactions in nine-spined stickleback body size and growth (Välimäki & Herczeg, 2012). Likewise, earlier studies have reported habitat-dependent population divergence in nine-spined stickleback brain anatomy: marine sticklebacks have larger *bulbi olfactorii* and *telencephala* than pond fish (Gonda *et al.*, 2009a). The link between brain size and cognition has been supported both between and within species (Sol *et al.*, 2005; Kotrschal *et al.*, 2013). However, the observed lack of sex × habitat interactions in brain anatomy in this study implies that the selective forces shaping population divergence in brain size and/or cognition act on the sexes similarly. With other words, sex-specific selection forces on brains and cognitive functions appear to differ only negligibly between pond and marine habitats. We note that the number of populations in our study was low, focusing only on variation in particular environmental factors; hence, we cannot exclude the possibility that population variation in brain sexual dimorphism might be present along different environmental gradients.

Apart from local adaptation, phenotypic differentiation between populations is influenced also by direct environmental effects, as well interactions between genetic and environmental effects. Here, we used common garden experiment to control for environmental influences and also elucidated possible interaction effects by repeating the experiments under different treatment conditions. Inclusion of the different treatments is particularly relevant for this investigation as a sex × food treatment interaction in nine-spined stickleback feeding behaviour was uncovered in a previous study (Herczeg & Välimäki, 2011). Likewise, rearing environment effects on nine-spined stickleback brain development were already known: the presence of conspecifics resulted in enlarged *tectum opticum*, whereas perceived predation risk affected *bulbus olfactorius* and *hypothalamus* development (Gonda *et al.*, 2009b, 2012).

Here, we found only a marginally significant sex × food treatment interaction: there was female-biased sexual size dimorphism in *bulbus olfactorius* (13.14%) in the high food treatment, but not in the low food treatment. *Bulbus olfactorius* has been found to be the most variable brain part in the nine-spined stickleback so far (Gonda *et al.*, 2009a,b, 2012). Hence, *bulbus olfactorius* size might have strong fitness consequences. The reason for the pattern observed in the present study is unclear, but it suggests that sexes not only differ in general brain size/architecture, but also that the brain development of the two sexes is affected differently by the same environmental factors. A somewhat similar effect (sex × social environment interaction on brain size) was reported by Kotrschal *et al.* (2012b) studying guppies, further supporting the conjecture that sex effects on the brain phenotypes in nature can be environment dependent.

In summary, the results demonstrate strong sexual dimorphism in brain size and architecture in the nine-spined stickleback. The relatively larger brains, *telencephala, cerebella* and *hypothalami* (difference ranging between 6.15 and 16.44%) in males than in females

© 2014 THE AUTHORS. *J. EVOL. BIOL.* **27** (2014) 1604–1612
JOURNAL OF EVOLUTIONARY BIOLOGY © 2014 EUROPEAN SOCIETY FOR EVOLUTIONARY BIOLOGY

might be explainable by the high male investment into reproductive behaviour and parental care as compared to females. Further, the lack of sexual divergence in body size–brain size allometry suggests that sexual dimorphism in brain size and architecture is not simply a consequence of the sexual size dimorphism reported from the species earlier (Herczeg *et al.*, 2010; Välimäki & Herczeg, 2012). Although previous studies also found signs of local adaptation in brain morphology and even in brain plasticity, we did not find any sex-dependent habitat effect and only a trend for sex-dependent *bulbus olfactorius* plasticity. All in all, sex seems to be an important determinant of brain anatomy, and perhaps brain plasticity too. However, assessment of the biological significance of the reported sexual dimorphism requires more fine-tuned studies linking brain anatomy changes and to cognitive (or other fitness related) functions.

Acknowledgments

We are highly indebted to Göran Englund, Jacquelin DeFaveri, Jenni Hämäläinen, Lotta Kvarnemo, Tuomas Leinonen, Pia Rännänen and Pirkko Siikamäki for their help in obtaining the samples, and Mirva Turtiainen, Linda Uoti, Maarit Haataja, Nurul Izza Ab Ghani, Riikka Kaartinen, Joona Lehtomäki and Marika Lilja for their help in fish maintenance. Thanks to John Loehr for correcting the English. We thank two anonymous reviewers for their valuable comments. Our work was supported by the Academy of Finland (GH: #128716; JM: #s 250435, 263722 & 265211), the Hungarian Scientific Research Fund (GH # OTKA-K 105517), the János Bólyai Research Scholarship of the Hungarian Academy of Sciences (GH), Finnish Graduate School in Population Genetics, Ella & Georg Ehrnrooth Foundation, Eemil Aaltonen Foundation (KV) and Finnish School in Wildlife Biology, Conservation and Management (AG). The experiment was conducted under the licence of the Finnish National Animal Experiment Board (Licence number STH223A).

References

Ab Ghani, N.I., Herczeg, G. & Merilä, J. 2012. Body size divergence in nine-spined sticklebacks: disentangling additive genetic and maternal effects. *Biol. J. Linn. Soc.* **107**: 521–528.

Ab Ghani, N.I., Herczeg, G., Leinonen, T. & Merilä, J. 2013. Evidence for genetic differentiation in timing of maturation among nine-spined stickleback populations. *J. Evol. Biol.* **24**: 775–782.

Ådjers, K., Appelberg, M., Eschbaum, R., Lappalainen, A., Minde, A., Repecka, R. *et al.* 2006. Trends in coastal fish stocks of the Baltic Sea. *Boreal Env. Res.* **11**: 13–25.

Aiello, L.C. & Wheeler, P. 1995. The expensive tissue hypothesis – The brain and digestive system in human and primate evolution. *Curr. Anthropol.* **36**: 199–221.

Aikio, S., Herczeg, G., Kuparinen, A. & Merilä, J. 2013. Optimal growth strategies under divergent predation pressure. *J. Fish Biol.* **82**: 318–331.

Allen, K.L. & Kay, R.F. 2012. Dietary quality and encephalization in platyrrhine primates. *Proc. R. Soc. B* **279**: 715–721.

Aviles, J.M. & Garamszegi, L.Z. 2007. Egg rejection and brain size among potential hosts of the common cuckoo. *Ethology* **113**: 562–572.

Barton, R.A. & Capellini, I. 2012. Maternal investment, life history, and the costs of brain growth in mammals. *Proc. Natl. Acad. Sci. USA* **108**: 6169–6174.

Bell, M.A. & Foster, S.A. (eds) 1994. *The Evolutionary Biology of Threespine Stickleback*. Oxford University Press, Oxford, UK.

Benjamini, Y. & Hochberg, Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. Roy. Stat. Soc. B* **57**: 289–300.

Broglio, C., Rodriguez, F. & Salas, C. 2003. Spatial cognition and its neural basis in teleost fish. *Fish Fish.* **4**: 247–255.

Crispo, E. & Chapman, L.J. 2010. Geographic variation in phenotypic plasticity in response to dissolved oxygen in an African cichlid fish. *J. Evol. Biol.* **23**: 2091–2103.

Day, L.B., Westcott, D.A. & Olster, D.H. 2005. Evolution of bower complexity and cerebellum size in bowerbirds. *Brain Behav. Evol.* **66**: 62–72.

Fitzpatrick, J.L., Almbro, M., Gonzalez-Voyer, A., Hamada, S., Pennington, C., Scanlan, C. *et al.* 2012. Sexual selection uncouples the evolution of brain and body size in pinnipeds. *J. Evol. Biol.* **25**: 1321–1330.

Garamszegi, L.Z., Eens, M., Erritzøe, J. & Møller, A.P. 2005a. Sperm competition and sexually size dimorphic brains in birds. *Proc. R. Soc. B* **272**: 159–166.

Garamszegi, L.Z., Eens, M., Erritzøe, J. & Møller, A.P. 2005b. Sexually size dimorphic brains and song complexity in passerine birds. *Behav. Ecol.* **16**: 335–345.

Gonda, A., Herczeg, G. & Merilä, J. 2009a. Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? *J. Evol. Biol.* **22**: 1721–1726.

Gonda, A., Herczeg, G. & Merilä, J. 2009b. Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain. *Proc. R. Soc. B* **276**: 2085–2092.

Gonda, A., Herczeg, G. & Merilä, J. 2011. Population variation in brain size of nine-spined sticklebacks (*Pungitius pungitius*) - local adaptation or environmentally induced variation? *BMC Evol. Biol.* **11**: 75.

Gonda, A., Välimäki, K., Herczeg, G. & Merilä, J. 2012. Brain development and predation: plastic responses depend on evolutionary history. *Biol. Lett.* **8**: 249–252.

Gonzalez-Voyer, A. & Kolm, N. 2010. Sex, ecology and the brain: evolutionary correlates of brain structure volumes in Tanganyikan Cichlids. *PLoS ONE* **5**: e14355.

Gonzalez-Voyer, A., Winberg, S. & Kolm, N. 2009a. Social fishes and single mothers: brain evolution in African cichlids. *Proc. R. Soc. B* **276**: 161–167.

Gonzalez-Voyer, A., Winberg, S. & Kolm, N. 2009b. Distinct evolutionary patterns of brain and body size during adaptive radiation. *Evolution* **63**: 2266–2274.

de Groof, G., Verhoye, M., Poirier, C., Leemans, A., Eens, M., Darras, V.M. *et al.* 2009. Structural changes between seasons in the songbird auditory forebrain. *J. Neurosci.* **29**: 13557–13565.

Harvey, P.H., Clutton-Brock, T.H. & Mace, G.M. 1980. Brain size and ecology in small mammals and primates. *Proc. Natl. Acad. Sci. USA* **77**: 4387–4389.

Herczeg, G. & Välimäki, K. 2011. Intraspecific variation in behaviour: effects of evolutionary history, ontogenetic experience and sex. *J. Evol. Biol.* **24**: 2434–2444.

Herczeg, G., Gonda, A. & Merilä, J. 2009a. Evolution of gigantism in nine-spined sticklebacks. *Evolution* **63**: 7–27.

Herczeg, G., Gonda, A. & Merilä, J. 2009b. Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *J. Evol. Biol.* **22**: 544–552.

Herczeg, G., Gonda, A. & Merilä, J. 2009c. The social cost of shoaling covaries with predation risk in nine-spined stickleback (*Pungitius pungitius*) populations. *Anim. Behav.* **77**: 575–580.

Herczeg, G., Gonda, A. & Merilä, J. 2010. Rensch's rule inverted: female-driven gigantism in nine-spined stickleback *Pungitius pungitius*. *J. Anim. Ecol.* **79**: 581–588.

Herczeg, G., Gonda, A., Kuparinen, A. & Merilä, J. 2012. Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? *Evol. Ecol.* **26**: 109–122.

Huber, R., van Staaden, M., Kaufman, L.S. & Liem, K.F. 1997. Microhabitat use, trophic patterns and the evolution of brain structure in African cichlids. *Brain Behav. Evol.* **50**: 167–182.

Jacobs, L.F. 1996. Sexual selection and the brain. *Trends Ecol. Evol.* **11**: 82–86.

Kahilainen, K., Malinen, T., Tuomaala, A. & Lehtonen, H. 2004. Diel and seasonal habitat and food segregation of three sympatric *Coregonus lavaretus* forms in a subarctic lake. *J. Fish Biol.* **64**: 418–434.

Kihslinger, R.L. & Nevitt, G.A. 2006. Early rearing environment impacts cerebellar growth in juvenile salmon. *J. Exp. Biol.* **209**: 504–509.

Kihslinger, R.L., Lema, S.C. & Nevitt, G.A. 2006. Environmental rearing conditions produce forebrain differences in wild Chinook salmon *Oncorhynchus tshawytscha*. *Comp. Biochem. Physiol. A.* **145**: 145–151.

Koli, L. 1990. *Suomen Kalat*. Werner Söderström, Helsinki, Finland.

Koli, L., Rask, M., Viljanen, M. & Aro, E. 1988. The diet of perch, *Perca fluviatilis* L., at Tvärminne, Northern Baltic Sea, and a comparison with two lakes. *Aqua Fennica* **18**: 185–191.

Kolm, N., Gonzalez-Voyer, A., Brelin, D. & Winberg, S. 2009. Evidence for small scale variation in the vertebrate brain: mating strategy and sex affect brain size and structure in wild brown trout (*Salmo trutta*). *J. Evol. Biol.* **22**: 2524–2531.

Kotrschal, K., Van Staaden, M.J. & Huber, R. 1998. Fish brains: evolution and environmental relationships. *Rev. Fish Biol. Fish.* **8**: 373–408.

Kotrschal, A., Räsänen, K., Kristjansson, B.K., Senn, M. & Kolm, N. 2012a. Extreme sexual brain size dimorphism in sticklebacks: a consequence of the cognitive challenges of sex and parenting? *PLoS ONE* **7**: e30055.

Kotrschal, A., Rogell, B., Maklakov, A.A. & Kolm, N. 2012b. Sex-specific plasticity in brain morphology depends on social environment of the guppy, *Poecilia reticulata*. *Behav. Ecol. Sociobiol.* **66**: 1485–1492.

Kotrschal, A., Rogell, B., Bundsen, A., Svensson, B., Zajitschek, S., Brännström, I. *et al.* 2013. Artificial selection on relative brain size in the guppy reveals costs and benefits of evolving a larger brain. *Curr. Biol.* **23**: 1–4.

Kulczykowska, E. & Sánchez Vázquez, F.J. 2010. Neurohormonal regulation of feed intake and response to nutrients in fish: aspects of feeding rhythm and stress. *Aquacultural Res.* **41**: 654–667.

Laming, P.R. & McKinley, S.J. 1990. Habituation in goldfish (*Carassius auratus*) is impaired by increased interstimulus interval, interval variability, and telencephalic ablation. *Behav. Neurosci.* **104**: 869–875.

Lisney, T.J., Bennett, M.B. & Collin, S.P. 2007. Volumetric analysis of sensory brain areas indicates ontogenetic shift in the relative importance of sensory system in elasmobranch. *Raffl. Bull. Zool.* **14**: 7–15.

Madden, J. 2001. Sex, bowers and brains. *P. Proc. R. Soc. B* **268**: 833–838.

Merilä, J. 2013. Nine-spined stickleback (*Pungitius pungitius*): an emerging model for evolutionary biology research. *Ann. N. Y. Acad. Sci.* **1289**: 18–35.

Milinski, M. & Bakker, T.C.M. 1992. Costs influence sequential mate choice in sticklebacks, *Gasterosteus aculeatus*. *P. Proc. R. Soc. B* **250**: 229–233.

Mohammed, A.H., Zhu, S.W., Darmopil, S., Hjerling-Leffler, J., Ernfors, P., Winblad, B., Diamond, M.C., Eriksson, P.S. & Bogdanovic, N. 2002. Environmental enrichment and the brain. *Prog. Brain Res.* **138**: 109–133.

Møller, A.P. 2010. Brain size, head size and behaviour of a passerine bird. *J. Evol. Biol.* **23**: 625–635.

Navaterre, A., van Schaik, C.P. & Isler, K. 2011. Energetics and evolution of human brain size. *Nature* **480**: 91–93.

Nottebohm, F. & Arnold, A.P. 1976. Sexual dimorphism in vocal control areas of songbird brain. *Science* **194**: 211–213.

Östlund-Nilsson, S., Mayer, I. & Huntingford, F.A. 2007. *Biology of the Three-Spined Stickleback*. CRC Press, Boca Raton.

Park, P.J. & Bell, M.A. 2010. Variation of telencephalon morphology of the threespine stickleback (*Gasterosteus aculeatus*) in relation to inferred ecology. *J. Evol. Biol.* **23**: 1261–1277.

Pitnick, S., Jones, K.E. & Wilkinson, G.S. 2006. Mating system and brain size in bats. *Proc. R. Soc. B* **273**: 719–724.

Pollen, A.A., Dobberfuhl, A.P., Scace, J., Igulu, M.M., Renn, S.C.P., Shumway, C.A. *et al.* 2007. Environmental complexity and social organization sculpt the brain in Lake Tanganyikan cichlid fish. *Brain Behav. Evol.* **70**: 21–39.

Portavella, M., Salas, C., Vargas, J.P. & Papini, M.R. 2003. Involvement of telencephalon in spaced-trial avoidance learning in the goldfish (*Carassius auratus*). *Physiol. Behav.* **80**: 49–56.

Ratcliffe, J.M., Fenton, M.B. & Shettleworth, S.J. 2006. Behavioral flexibility positively correlated with relative brain volume in predatory bats. *Brain Behav. Evol.* **67**: 165–176.

Rohlf, F.J. 2006. *TPS software series*. Distributed by the author. Department of Ecology and Evolution, State University of New York, Stony Brook, NY.

Roth, T.C. & Pravosudov, V.V. 2009. Hippocampal volumes and neuron numbers increase along a gradient of environmental harshness: a large-scale comparison. *Proc. R. Soc. B* **276**: 401–406.

Roth, T.C., LaDage, L.D. & Pravosudov, V.V. 2012. Variation in hippocampal morphology along an environmental gradient: controlling for the effect of day length. *Proc. R. Soc. B* **278**: 2662–2667.

Sol, D., Duncan, R.P., Blackburn, T.M., Cassey, P. & Lefebre, L. 2005. Big brains, enhanced cognition, and response of birds to novel environments. *Proc. Natl. Acad. Sci. USA* **102**: 5460–5465.

Striedter, G.F. 2005. *Principles of Brain Evolution*. Sinauer Associates, Sunderland.
Välimäki, K. & Herczeg, G. 2012. Ontogenetic and evolutionary effects of predation and competition on nine-spined stickleback (*Pungitius pungitius*) body size. *J. Anim. Ecol.* **81**: 859–867.
Van Praag, H., Kempermann, G. & Cage, F.H. 2000. Neural consequences of environmental enrichment. *Nat. Rev.* **1**: 191–198.
Warren, D.L. & Iglesias, T.L. 2012. No evidence for the 'expensive tissue hypothesis' from an intraspecific study of a highly variable species. *J. Evol. Biol.* **25**: 1226–1231.
West-Eberhard, M.J. 2003. *Developmental Plasticity and Evolution*. Oxford University Press, Oxford.
White, S.A. & Fernald, R.D. 1993. Gonadotropin releasing hormone-containing neurons change size with reproductive state in female *Haplochromis burtoni*. *J. Neurosci.* **13**: 434–441.
de Winter, D. & Oxnard, C.E. 2001. Evolutionary radiation and convergences in the structural organization of mammalian brains. *Nature* **409**: 710–714.
Wootton, R.J. 1976. *The Biology of Sticklebacks*. Academic Press, New York.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Lack of sex-specific body size–brain size allometry illustrated with body weight as the body size proxy.
Table S1 Raw means and standard deviations of the variables analysed in the present study grouped by sex and population.

Data deposited at Dryad: doi:10.5061/dryad.27vh0

Received 18 November 2013; revised 4 April 2014; accepted 13 April 2014

doi:10.1111/evo.12268

BRINGING HABITAT INFORMATION INTO STATISTICAL TESTS OF LOCAL ADAPTATION IN QUANTITATIVE TRAITS: A CASE STUDY OF NINE-SPINED STICKLEBACKS

M. Karhunen,[1,2] O. Ovaskainen,[1] G. Herczeg,[1,3] and J. Merilä[1]

[1] *Department of Biosciences, University of Helsinki, PO Box 65, FI-00014, Finland*

[2] *E-mail: markku.karhunen@helsinki.fi*

[3] *Current Address: Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/c, H-1117, Budapest, Hungary*

Received May 2, 2013
Accepted August 21, 2013
Data Archived: Dryad doi: 10.5061/dryad.sg546

Detection of footprints of historical natural selection on quantitative traits in cross-sectional data sets is challenging, especially when the number of populations to be compared is small and the populations are subject to strong random genetic drift. We extend a recent Bayesian multivariate approach to differentiate between selective and neutral causes of population differentiation by the inclusion of habitat information. The extended framework allows one to test for signals of selection in two ways: by comparing the patterns of population differentiation in quantitative traits and in neutral loci, and by comparing the similarity of habitats and phenotypes. We illustrate the framework using data on variation of eight morphological and behavioral traits among four populations of nine-spined sticklebacks (*Pungitius pungitius*). In spite of the strong signal of genetic drift in the study system (average F_{ST} = 0.35 in neutral markers), strong footprints of adaptive population differentiation were uncovered both in morphological and behavioral traits. The results give quantitative support for earlier qualitative assessments, which have attributed the observed differentiation to adaptive divergence in response to differing ecological conditions in pond and marine habitats.

KEY WORDS: Adaptation, F_{ST}, G matrix, genetic drift, population differentiation, *Pungitius pungitius*.

The study of ultimate and proximate determinants of population differentiation in traits of ecological importance—both in spatial and temporal contexts—is central to contemporary evolutionary biology research. Attempts to identify, understand, and infer selection pressures responsible for local adaptation in various taxa are vigorous areas of research (Kawecki and Ebert 2004; Leinonen et al. 2008; Hereford 2009; Nosil et al. 2009; Blanquart et al. 2012). Similarly, much research and debate has been centering around reconciling whether observed phenotypic changes in mean values of phenotypic traits over time represent evolutionary responses to natural selection, or merely plastic phenotypic responses to changing environmental conditions (Gienapp et al. 2008; Kuparinen and Merilä 2008; Pujol et al. 2008; Brommer 2011).

Apart from the problem of differentiating between genetic and environmental causes of population differentiation, evolutionary studies are also faced with the problem of distinguishing between adaptive and "neutral" explanations for observed divergence. Although methods for inferring action of natural selection at the molecular level keep on developing and have found many uses in evolutionary biology (Egea et al. 2008; Foll and Gaggiotti 2008; Coop et al. 2010; Brommer 2011; Narum and Hess 2011), their utility is limited in the sense that they normally cannot be used to detect selection on quantitative traits. One of the main reasons for this is the highly polygenic basis of quantitative traits, and the fact that the magnitude of differentiation at individual coding loci is not expected to differ much from that of the neutral loci (McKay and Latta 2002; Le Corré and Kremer 2012). To this end, $Q_{ST} - F_{ST}$ comparisons (e.g., Merilä and Crnokrak 2001; Whitlock and Guillaume 2009; Leinonen et al. 2013) have provided a practical way to infer action of natural selection on quantitative traits. In brief, if Q_{ST}, an index of divergence in quantitative traits (Spitze 1993), exceeds F_{ST}, a measure of random genetic drift estimable from neutral molecular markers (Rousset 2002), there is evidence for the action of diversifying natural selection.

Although judicious application of the $Q_{ST} - F_{ST}$ comparisons can provide a good platform to detect signatures of natural selection (e.g., Rhone et al. 2010; Leinonen et al. 2013), the method has various limitations. Among other things, its standard implementation requires a fairly large number of populations to be compared in order to have a reasonable statistical power to pick up signatures of selection (O'Hara and Merilä 2005; Ovaskainen et al. 2011). Another issue is that the method's power to discriminate between natural selection and random genetic drift is compromised when the baseline level of "neutral" differentiation, that is F_{ST}, becomes high (Hendry 2002). Both of these shortcomings are circumvented by the method of Ovaskainen et al. (2011), which has been as yet little used because of its computational complexity and lack of ready-to-use scripts to implement it—a situation which has recently changed (Karhunen et al. 2013). Nevertheless, the method has been only applied to one case study so far (Karhunen et al. 2013), and even then, merely for illustrative purposes.

Both $Q_{ST} - F_{ST}$ comparisons and the method of Ovaskainen et al. (2011) ignore habitat information and focus only on demographic processes, and in the case of the latter, on genetic correlations among traits (Ovaskainen et al. 2011). However, given that random processes such as genetic drift are not expected to result in independent evolution of similar phenotypes in different localities (e.g., Schluter 2000; Langerhans and DeWitt 2004), also habitat information is likely to carry useful information about causes of population differentiation. Hence, the analytical framework of Ovaskainen et al. (2011) could be improved by developing it to account for habitat information and its correlation with observed phenotypes. In other words, it should—at least sometimes—be possible to enhance the power to detect the footprints of selection in the framework of Ovaskainen et al. (2011) by developing a new statistical test which would incorporate data on environmental variables.

Pond populations of the nine-spined stickleback (*Pungitius pungitius*) provide an interesting and challenging case—both biologically and methodologically—to detect footprints of natural selection in the past. They have evolved similar morphological (Herczeg et al. 2009a, 2010; Ab Ghani et al. 2012; Välimäki et al. 2012), life history (Herczeg et al. 2012; Ab Ghani et al. 2013), behavioral (Herczeg et al. 2009b,c, 2013; Herczeg and Välimäki 2011; Ab Ghani et al. 2012), neuroanatomical (Gonda et al. 2009a,b, 2012; Trokovic et al. 2011, 2012), and physiological (Waser et al. 2010) phenotypes independently in different Fennoscandian localities. Common-garden experiments have shown that the observed differentiation has a genetic basis for many of the studied traits (Ab Ghani et al. 2012, 2013; Herczeg et al. 2012). At the same time, the pond populations are subject to severely reduced neutral genetic variability and strong genetic drift (Shikano et al. 2010; Bruneaux et al. 2013) which poses challenges for detecting signals of natural selection by $Q_{ST} - F_{ST}$ type of methods (Hendry 2002). Therefore, standard $Q_{ST} - F_{ST}$ comparisons are unlikely to work here (but see Shimada et al. 2011 for a contrary example), or at least, they are expected to have a very low statistical power to do so. Similar problems will be faced in many other contexts too, and a good case in point is provided by the contrast between freshwater and marine fishes in general: due to their restricted gene flow and smaller effective population sizes, freshwater fish show much more population structuring in neutral marker genes than marine fish (Ward et al. 1994; DeWoody and Avise 2000; DeFaveri et al. 2012). Hence, as indicated by recent theoretical treatments (Kronholm et al. 2010; Le Corré and Kremer 2012), $Q_{ST} - F_{ST}$ comparisons are unlikely to be of much help in distinguishing neutral and selective differentiation in these systems.

The main aims of this study were twofold. First, to extend the method of Ovaskainen et al. (2011) to detect footprints of natural selection in quantitative traits by introducing a new statistical test (H test) to infer whether populations found in similar habitats are more similar to each other than expected by chance, that is on the basis of their shared evolutionary history and genetic correlations among traits. Unlike the hierarchical $Q_{ST} - F_{ST}$ comparisons (Chapuis et al. 2008; Whitlock and Gilbert 2012), the method developed here does not require the habitats to fall in discrete classes or types. Instead, they may be described through a set of both continuous and categorical variables. Second, using this extended analytical framework, we looked for signatures of habitat-specific diversifying selection in five morphometric and three behavioral traits in nine-spined sticklebacks. To this end, we are able to provide the first formal demonstration of natural selection (but see Shimada et al. 2011) in this extensively studied model system, where earlier studies have provided only indirect indications for adaptive differentiation in a range of quantitative traits (reviewed by Merilä 2013).

Methods

STUDY SPECIES AND POPULATIONS

The nine-spined stickleback is distantly related to its better-known congener—the three-spined stickleback (*Gasterosteus*

Figure 1. Map showing the location of study sites. The fish used as the parental generation in the common-garden study originate from the following populations: BAS = Baltic Sea, WHS = White Sea, PYÖ = Pyöreälampi, and BYN = Bynästjärnen. BYN and PYÖ represent independent postglacial colonizations from BAS and WHS, respectively. Closed dots denote marine habitats and open dots denote ponds.

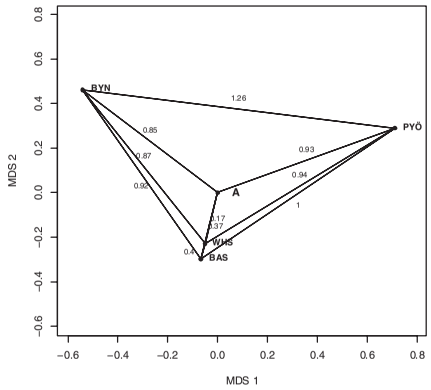


Figure 2. The pattern of genetic differentiation inferred from neutral genetic markers. The local populations were mapped into a 2D coordinate system by using multidimensional scaling. The numbers marked along the lines indicate genetic distances between the populations, measured in units of ancestral SD of a neutral trait (see Karhunen et al. 2013 and main text). The pattern of phenotypic differentiation is expected to be similar under random genetic drift. The figure has been produced by modifying the code of viz.theta in driftsel (Karhunen et al. 2013). "A" refers to the unobserved ancestral population.

aculeatus)—but differs from it by being more widely distributed in freshwater habitats (Wootton 1984). It also shows much more genetic structuring in neutral marker genes over comparable ranges both in freshwater and marine habitats than the three-spined stickleback (DeFaveri et al. 2012). In spite of being subject to strong random genetic drift, studies of Fennoscandian freshwater populations of the nine-spined stickleback have uncovered evidence for widespread environmental adaptation (Merilä 2013).

The populations included into this study comprise of two sea (Helsinki, Baltic Sea, 60°13′N; 25°11′E and Levin Navolok Bay, White Sea, 66°18′N; 33°25′E) and two pond (Åmsele, Bynästjärnen, 64°27′N; 19°26′E and Kuusamo, Pyöreälampi 66°15′N; 29°26′E) populations (Fig. 1). These populations were chosen to represent two independent postglacial colonizations of freshwater habitats from two different ancestral marine populations (Shikano et al. 2010; Bruneaux et al. 2013).

THE DATA

A total of 92 F_1-generation fish born to wild-collected parents were reared in a common-garden environment in the aquaculture facilities of the University of Helsinki from June–July 2007 to March–April 2008. These data have earlier been used for instance by Herczeg et al. (2009a,b; 2012), and the detailed rearing procedures have been described in these publications: For each population, five full-sib families were produced using wild-caught parental fish, that is five independent parental fish of each sex from each population. The offspring were reared from hatching until the age of 256 days, of which 92 individuals (Baltic Sea = 21; White Sea = 24; Bynastjärnen = 22; Pyöreälampi = 25) were chosen at random to be used in this study, whereas the rest were used in neuroanatomical studies (Gonda et al. 2009a). At the end of the experiment, the fish were killed with an overdose of tricaine methanosulphonate, digital photographs were taken from the left side of each fish, and five morphological traits were measured from the photographs, using landmark coordinates and basic Euclidean geometry. For the description of landmarks, see Figure 2 in Herczeg et al. (2010). The five morphological traits included in this study are: standard length (distance between landmarks 1 and 8; a proxy of body size), body depth (distance between landmarks 4 and 12), head length (distance between landmarks 1 and 13), pelvic girdle length (distance between landmarks "X" and 4), and caudal peduncle length (distance between landmarks 6 and 7. All measurements were taken with programs tpsDig 2 (Rohlf 2008) and tpsRelw 1.46 (Rohlf 2007).

Table 1. Summary of raw data. Reported are the mean (± SE) phenotypic values in the offspring (F_1) generation of the common-garden study. Feeding denotes the binary decision to feed or not during a 300 sec experimental time; thus, the population means of feeding can to be interpreted as probabilities. The right-hand columns (R^2) show the proportion of phenotypic variation explainable by habitat and population, respectively. These were obtained from a conventional ANOVA model with sex, habitat, and population (nested within habitat) as explanatory variables. n = number of individuals.

Trait	Baltic Sea ($n = 21$)	White Sea ($n = 24$)	Bynästjärnen ($n = 22$)	Pyöreälampi ($n = 25$)	R^2 of habitat (%)	R^2 of population (%)
Morphology						
Standard length (mm)	46.5 (±1.0)	60.4 (±0.8)	68.7 (±0.8)	72.1 (±1.3)	50	31
Body depth (mm)	8.7 (±0.2)	10.8 (±0.2)	12.9 (±0.1)	13.0 (±0.2)	57	26
Head length (mm)	10.1 (±0.1)	12.0 (±0.1)	14.1 (±0.1)	15.7 (±0.1)	39	53
Pelvic girdle length (mm)	6.7 (±0.2)	8.2 (±0.1)	6.8 (±0.2)	8.3 (±0.2)	0	39
Caudal peduncle length (mm)	7.0 (±0.3)	10.3 (±0.3)	10.6 (±0.2)	10.1 (±0.4)	36	11
Behavior						
Aggression (number)	4.6 (±2.0)	4.0 (±1.6)	9.1 (±1.7)	15.6 (±2.0)	3	24
Risk taking 1 (sec)	273 (±61)	438 (±49)	115 (±30)	119 (±31)	5	29
Risk taking 2 (binary)	0.22 (±0.10)	0.23 (±0.09)	0.74 (±0.11)	0.86 (±0.08)	12	22

The three behavioral traits, taken from same individuals and earlier analyzed by Herczeg et al. (2009b), were the following: number of attacks against a stimulus conspecific (henceforth: "aggressiveness"), time till the fish fully came out from a refuge in a new, potentially dangerous situation (henceforth: "risk taking 1"), and time till the first biting attempt on familiar food after a simulated attack (henceforth: "risk taking 2"). However, the distribution of "risk taking 2" was found to be highly skewed, with almost half of the fish not biting at all during the 300-s-long trials, and most of the rest biting during the first few seconds. Thus, we coded "risk taking 2" as a binary variable such that value 1 was assigned for the fish that tried to bite in <300 s, and value 0 was used otherwise. The raw data are summarized in Table 1.

We note that although driftsel analyses—including the new H test introduced later—can be in principle performed using wild-collected (i.e., purely phenotypic) data (Karhunen et al. 2013, p. 747), results of such analyses should be interpreted with caution as they do not differentiate between environmental and genetic influences on phenotypes. However, as our analyses are based on common garden reared fish, we assume that our estimates of population differentiation are reflective of genetic differences among populations.

To obtain estimates of neutral genetic differentiation, the genetic variability of 12 unlinked microsatellite markers was assessed using the R package RAFM (Karhunen and Ovaskainen 2012). These data derive from Shikano et al. (2010), and comprise the genotypes of 183 individuals (Baltic Sea = 40, White Sea = 40, Bynastjärnen = 40, Pyöreälampi = 63) in the following loci: 1125P, 4174P, 7033P, Stn49, Stn96, Stn100, Stn130, Stn163, Stn173, Stn196, Stn198, and Stn380. Data on genetic variability and full characterization of microsatellite marker data can be found from Shikano et al. (2010). All these data have been uploaded to Dryad doi: 10.5061/dryad.sg546.

STATISTICAL METHODS

We analyzed the data with the R package driftsel (Karhunen et al. 2013), which we extend in this study to accommodate binary traits (see Appendix A and Fig. A1) and habitat information. The updated version of driftsel that implements the new statistical test is available at http://www.helsinki.fi/biosci/egru/software/. The new version has been also uploaded to CRAN (http://cran.r-project.org/).

driftsel delivers the joint posterior distribution for a number of parameters, including the mean additive genotypes of the local populations ("population means"), the unobserved ancestral means, and the ancestral **G** matrix. Their joint distribution can then be postprocessed in driftsel to calculate the signal of selection S (Ovaskainen et al. 2011). This figure is interpreted such that $S = 0.5$ implies perfect match with random genetic drift, whereas $S < 0.05$ implies stabilizing natural selection at a 95% credibility level, and $S > 0.95$ implies divergent natural selection at this credibility level. This statistic takes into account the pattern of relatedness among local populations as well as the ancestral genetic correlations, but does not use habitat information. Therefore, we develop here a new test statistic H which has a similar interpretation as S, but takes into account also the correlation between genotypes and habitats. In a nutshell, the test statistic H measures the similarity between the distribution of quantitative traits and the distribution of environmental conditions, taking into account the fact that populations found in similar habitats may have a shared phylogenetic history. We will explain the derivation of H later.

In this particular case of the nine-spined stickleback, we use a single categorical variable to describe the habitats, that is we label the habitats as either pond or marine, but the developed method can use an arbitrary number of both discrete and continuous environmental variables. However, it should be noted that once the number of environmental variables to be analyzed increases, typical problems associated with multiple testing arise. Thus, the method will require larger number of populations to have sufficient statistical power to pick up similarity between the distribution of quantitative traits and the distribution of the individual environmental variables.

The derivation of the test statistic H is based on the following ideas: we first calculate an environmental distance matrix between each pair of local populations. We then develop a measure for the similarity of this matrix and a corresponding matrix for the mean additive genotypes. We then ask whether this similarity measure has a value that is compatible with the null hypothesis of random genetic drift. This is obtained by simulating the distribution of the similarity measure under random genetic drift and by comparing the observed similarity value to this distribution.

The environmental distance matrix $\mathbf{D}^E$ is either provided directly by the user or calculated from a set of environmental covariates as

$$\mathrm{D}_{ij}^E = \sum_{k=1}^{d_E} (x_{ik} - x_{jk})^2, \tag{1}$$

where x_i is the vector of environmental covariates for population i, d_E is the number of environmental covariates, and the sum can be weighted to best reflect the a priori expectation of the relative importance of different habitat variables. We do not consider weighting in this study, as we have only one covariate for these data, that is the pond/marine indicator. The distance matrix $\mathbf{D}^T$ for population means of the quantitative traits is defined in analogy to equation (1) as

$$\mathrm{D}_{ij}^T = \sum_{k=1}^{d_T} \left(a_{ik}^{\mathcal{P}} - a_{jk}^{\mathcal{P}}\right)^2, \tag{2}$$

where $a_{ik}^{\mathcal{P}}$ and are the population means for trait k in populations i and j, respectively, and d_T is the number of traits. Again, different traits can be given different weights, but we proceed here with unweighted analysis. We compare the similarity of these two distance matrices by the Mantel test statistic

$$\mathrm{M}\left(\mathbf{D}^E, \mathbf{D}^T\right) = \sum_{i=1}^{n_{\mathcal{P}}} \sum_{j=1}^{i} \mathrm{D}_{ij}^E \mathrm{D}_{ij}^T, \tag{3}$$

where $n_{\mathcal{P}}$ denotes the number of populations. We note that $\mathrm{M}\left(\mathbf{D}^E, \mathbf{D}^T\right)$ is merely the product moment of the unique entries in $\mathbf{D}^E$ and $\mathbf{D}^T$. We do not calculate the usual P value associated with $\mathrm{M}\left(\mathbf{D}^E, \mathbf{D}^T\right)$ by using Mantel's (1967) permutation procedure; rather, we use $\mathrm{M}\left(\mathbf{D}^E, \mathbf{D}^T\right)$ as a summary statistic which we compare to a null model. As shown by Ovaskainen et al. (2011), the population means are expected to be distributed as

$$\mathbf{a}^{\mathcal{P}} \sim \mathrm{MVN}\left(\mu \otimes \mathbf{I},\ 2\mathbf{G}^{\mathcal{A}} \otimes \theta^{\mathcal{P}}\right) \tag{4}$$

subject to random genetic drift and a low mutation rate. Above, $\mathbf{a}^{\mathcal{P}}$ is the vector of population means, μ is the mean additive genotype in the ancestral population, $\mathbf{G}^{\mathcal{A}}$ is the ancestral variance–covariance matrix of the traits in question, $\otimes$ is a Kroenecker product, $\theta^{\mathcal{P}}$ is the matrix of population-level coancestry coefficients, and $\mathbf{I}$ is a unit vector of the same dimension as $\theta^{\mathcal{P}}$, all of which can be estimated by using driftsel (Karhunen et al. 2013).

To compare the observed value $\mathrm{M}(\mathbf{D}^E, \mathbf{D}^T)$ to that expected due to random genetic drift, we denote by $\mathbf{D}^{T,R}$ a matrix of trait distances randomized from the above distribution and calculated in analogy to equation (2). We then define the test statistic H as the probability that the randomized product moment $\mathrm{M}\left(\mathbf{D}^E, \mathbf{D}^{T,R}\right)$ falls behind its observed counterpart:

$$H := \mathrm{P}\left\{\mathrm{M}\left(\mathbf{D}^E, \mathbf{D}^T\right) > \mathrm{M}\left(\mathbf{D}^E, \mathbf{D}^{T,R}\right)\right\}. \tag{5}$$

Above in equation (5), the probability is to be understood in context of evolutionary stochasticity, that is random genetic drift, represented by equation (4). To account for parameter uncertainty, we average the test statistic H over the joint posterior distribution of the parameters $(\mathbf{a}^{\mathcal{P}}, \mu, \mathbf{G}^{\mathcal{A}}, \theta^{\mathcal{P}})$. We index the posterior sample (obtained by driftsel) by t, so that in practice t denotes the (thinned) Monte Carlo Markov Chain (MCMC) iteration round. Using this notation, H is evaluated by calculating the fraction of cases for which $\mathrm{M}(\mathbf{D}^E, \mathbf{D}_t^T) > \mathrm{M}(\mathbf{D}^E, \mathbf{D}_t^{T,R})$ holds, such that $\mathbf{D}_t^T$ is calculated based on the value of $\mathbf{a}_t^{\mathcal{P}}$, while $\mathbf{D}_t^{T,R}$is randomized from equation (4) based on the values of μ_t, $\mathbf{G}_t^{\mathcal{A}}$, and $\theta_t^{\mathcal{P}}$.

A value of H close to one implies that the distribution of population means is more similar with the distribution of environmental covariates than would be expected at random, that is on the basis of random genetic drift. This can be interpreted as a sign of local adaptation to the conditions described by the matrix $\mathbf{D}^E$. Calculation of H is implemented in driftsel 2.0 as H.test, whereas the old S statistic is implemented as S.test. We note that the reason why we do not perform the usual Mantel test based on permutation of populations is that the value of $\mathrm{M}\left(\mathbf{D}^E, \mathbf{D}^{T,R}\right)$ is often expected to be positive, because populations found in similar habitats often have a shared evolutionary history (in terms of the coancestry matrix $\Theta^{\mathcal{P}}$). This violates the assumptions of the usual Mantel (1967) test.

In addition to driftsel analyses, we also partition the variation of different traits into the contributions of habitat type and population identity by calculating the R^2 values for these two explanatory factors. This is done for purely illustrative purposes, to give an idea about the relative importance of these factors; no inferences can be drawn regarding the cause of differentiation by using this method. The R^2 values were calculated by fitting an ordinary linear model and performing the variance analysis, using lm and anova in R, respectively.

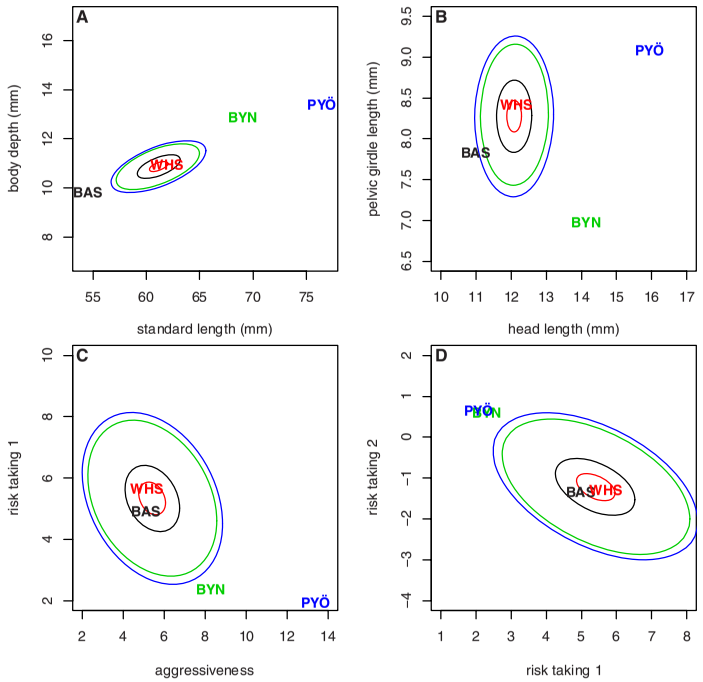


Figure 3. Pattern of phenotypic differentiation in selected traits. Panels A and B present two examples of morphological traits, whereas panels C and D present examples of behavioral traits. The population codes indicate the mean additive genotypes, whereas the ellipses of respective colors denote median distances of differentiation expected under random genetic drift, as inferred by driftsel. The ellipses are centered at the Bayesian estimate of ancestral mean genotype. This figure has been produced by modifying the code of viz.traits in driftsel. For interpretation of the behavioral traits, see main text.

Results

The overall level of neutral genetic differentiation among the four populations was found to be moderately high, $F_{ST} = 0.35$ (95% CI: 0.31–0.38). The pattern of neutral genetic differentiation (or inversely, interpopulation relatedness) is illustrated in Figure 2. The two sea populations (Baltic Sea and White Sea) appear very similar to each other and the unobserved ancestral population, whereas the pond populations (Bynästjärnen and Pyöreälampi) appear highly differentiated both from the sea populations and each other (Fig. 2). The edges of the graph show expected drift distances of a hypothetical neutral trait with unit ancestral variance, which can be calculated from the estimate of $\theta^{\mathcal{P}}$ alone (Karhunen et al. 2013). Hence, this pattern also provides the neutral expectation for phenotypic differentiation under random genetic drift, and it is implicitly used as a baseline pattern in the neutrality tests reported later.

Regarding the morphological traits, a very strong signal of diversifying natural selection was found both with and without habitat information ($H = 1.00$, $S = 1.00$) in a multivariate analysis with all five traits included. This finding was reinforced by a qualitative assessment based on visualization of the mean phenotypes (Fig. 3A, B). Although the evolution in morphological traits has occurred approximately along the lines of least resistance (Fig. 3A), the pond populations and the Baltic Sea population

Table 2. Signals of selection in behavioral traits. Test statistic *S* takes into account ancestral genetic correlations and the pattern of relatedness among local populations, whereas the *H* statistic takes also into account habitat information (in this case, pond/marine origin). Aggressiveness = no. of attacks against a stimulus conspecific, risk taking 1 = time till the fish fully came out from a refuge in a new, potentially dangerous situation; risk taking 2 = whether fish bit on food after a simulated attack within 300 sec. Statistically significant effects are in bold.

Traits analyzed	*S*	*H*
Aggressiveness + Risk taking 1 + Risk taking 2	0.91	**0.99**
Aggressiveness + Risk taking 1	0.93	**0.98**
Aggressiveness + Risk taking 2	0.89	**0.98**
Risk taking 1 + Risk taking 2	0.70	0.86
Aggressiveness	0.91	**0.97**
Risk taking 1	0.71	0.79
Risk taking 2	0.69	0.84

have evolved further away from the ancestral mean than would be expected under the neutral model (represented by the ellipses in Fig. 3A, B); thus, the clear signals (high *S* and *H*) of divergent natural selection. Note that in absence of ancestral correlation (Fig. 3B), the apparent line of least resistance is arbitrary and depends on the scaling of axes. The population means in Figure 3 do not exactly match the population means in Table 1, because the latter represent mean phenotypes, and thus include the influences of sex and rearing environment, whereas the values in Figure 3 represent mean additive genotypes, inferred from the data by the Bayesian approach.

The signal of selection in behavioral traits was clear-cut when using habitat information ($H = 0.99$ in a multivariate analysis with all traits included), but less so when ignoring it ($S = 0.91$; Table 1). This finding was in line with the observation that the proportions of variation explained by habitat and population are somewhat lesser for behavioral than for morphological traits (Table 1). Once the behavioral traits are investigated in more detail by analyzing different combinations or subset of traits, it becomes apparent that inclusion of habitat information (*H* test) increases the power to detect natural selection (Table 2). However, this finding is not to be interpreted so that the *H* test is unequivocally a more powerful method than the *S* test (see Discussion). Visual inspection of the patterns of divergence in behavioral traits (Fig. 3C, D) revealed them to be similar to those found in morphological traits (Fig. 3A, B), but at this time, both sea populations (WHS and BAS) had mean genotypes much compatible with random genetic drift. Thus, the signatures of selection in behavioral traits were weaker here.

Discussion

In this article, we have developed a new analytical metric to detect signatures of past natural selection on phenotypic traits, and illustrated the use of this metric into the case study of Fennoscandian nine-spined sticklebacks. Our results show that the stickleback populations have experienced divergent natural selection especially in morphological, but also in behavioral traits. As such, the results provide formal evidence for the assertion that the phenotypic divergence observed among nine-spined stickleback populations is adaptive, which has been previously difficult to demonstrate by using $Q_{ST} - F_{ST}$ comparisons, due to a high degree of neutral baseline differentiation in marker genes among these populations (Shikano et al. 2010; Bruneaux et al. 2013). To the new analytical metrics, the results demonstrate that incorporation of environmental information into driftsel's (Karhunen et al. 2013) neutrality test can help to filter out signatures of selection in situations where they would otherwise go undetected. In what follows, we will discuss these points in more detail.

The inclusion of habitat information in the neutrality test (the new *H* test) increased the ability of driftsel (Karhunen et al. 2013) to detect natural selection in some cases, particularly in the case of the behavioral traits. However, caution should be exercised in drawing general conclusions on the relative merits of *H* and *S*, as these two tests focus on fundamentally different aspects of information in the data. The *S* test asks whether the mean population genotypes have evolved differently from what can be expected regarding the pattern of coancestry among local populations. The *H* test asks whether the mean genotypes correlate with the environmental covariates more than expected on the basis of the pattern of coancestry among populations. As the test statistic *H* uses additional information about environmental covariates, it can in some instances uncover stronger indications of selection than the test statistic *S*. However, this does not need to be the case. For instance, if the pattern of phenotypic differentiation among populations is due to an unmeasured environmental gradient which is uncorrelated with the measured environmental variables, *H* will fail to indicate selection while *S* may still do so. Furthermore, the *H* test does not ask how far the populations have diverged from the ancestral mean, so that even a high degree of differentiation can pass unnoticed, as long as it is sufficiently uncorrelated with the environmental variation. Thus, we advocate the use of both tests. As such, the new *H* test can be viewed to add flexibility to hypothesis testing by allowing more rigorous tests of (adaptive) parallel phenotypic evolution in any kind of metric traits. Conceptually, the *H* test resembles the MANCOVA procedure of Langerhans and DeWitt (2004), which takes into account the effects of habitat on population on phenotypes, but does not involve a measure of the genetic similarity between populations. By incorporating information obtained from neutral DNA, we are

able to account explicitly for the influence of random genetic drift on the observed differentiation.

The *H* test developed here bears also some parallelism to genome scan studies devised to detect molecular footprints of natural selection (e.g., Storz 2005). These methods have evolved from generic scans for detecting signatures of selection, into approaches incorporating tests for association between selected, that is outlier, loci, and environmental variables (e.g., Coop et al. 2010; Tsumura et al. 2012). These tests are analogous to our *H* test in the sense that also the latter look for associations between environmental variables and footprints of selection. However, in contrast to *H* test, genome scan studies do not directly focus on phenotypes of interest, but markers putatively in linkage with the selected phenotypes. Nevertheless, analogously to the new generation of genome scan methods (reviewed by De Mita et al. 2013), the *H* test developed here can be viewed as an extension of the earlier *S* test (Ovaskainen et al. 2011), which in turn was built to improve traditional $Q_{ST} - F_{ST}$ approaches (see Leinonen et al. 2013).

A potential limitation of the methods used in this study is that equation (4), which forms the basis for both *S* and *H* tests, has been derived assuming that mutation rate is negligible with respect of other evolutionary and demographic forces (Ovaskainen et al. 2011). This may be a justified assumption in some cases (e.g., in case of a recently fragmented metapopulation), but we do not encourage the use of dritsel in studies which involve populations which have been subject to a very long history of reproductive isolation. We also emphasize the fact that the choice of neutral DNA markers used to estimate the degree of neutral baseline differentiation may matter (e.g., Edelaar et al. 2011): markers with extremely high mutation rates might lead into underestimation of differentiation expected due to genetic drift. A second technical limitation is that driftsel is, at present, limited to binary and normally distributed traits. Hence, further work is required, not only to explore the sensitivity of the method to its assumptions, but also to extend the sampling model by incorporating other distributional forms (e.g., Poisson and negative binomial), as many metric traits do not conform to simple normal or binomial models.

Although the earlier implementations of $Q_{ST} - F_{ST}$ comparisons typically require data from a large number of populations (O'Hara and Merilä 2005; Ovaskainen et al. 2011), the *H* and *S* tests can effectively use information also in small data sets. Indeed, in this study, strong statistical support for adaptive divergence was obtained using a very small data set comprising only 92 individuals from four populations only. Hence, apart from providing strong evidence for adaptive basis of population differentiation in nine-spined sticklebacks, our results highlight the virtues of *H* and *S* tests as a powerful and flexible approach for detecting natural selection.

Earlier studies of differentiation among marine and pond nine-spined stickleback populations have provided abundant evidence for strong, consistent, and genetically based differentiation in mean values of many morphological, life-history, neuroanatomical, and behavioral traits (reviewed in Merilä 2013). Although these differences are arguably likely to have evolved independently many times as adaptations to life in pond environments, the formal proof for natural selection being the agent behind these divergences has been lacking for all studied traits, except for body size (Shimada et al. 2011). Using the $Q_{ST} - F_{ST}$ approach, Shimada et al. (2011) showed that the body size differences between one pond and marine population exceed that to be expected on basis of genetic drift. However, the size divergence between these two populations was extreme and the conventional $Q_{ST} - F_{ST}$ approach was performing well. Here, by using the extended version of driftsel, we were able to provide formal evidence that natural selection is responsible for the divergence in body size, shape, armor, and behavior in a wider set of nine-spined stickleback populations.

In the case of the morphometric traits, the evidence for adaptive differentiation was obtained both by using *S* and *H* tests. However, in the case of the behavioral traits, *S* tests failed but the *H* test succeeded in finding statistically significant support for adaptive differentiation. This slight contrast in the results obtained for morphological and behavioral traits is consistent with the observation that habitat explains typically more of the variance in morphological traits than the population component (cf. Table 1), whereas the opposite was true in case of the behavioral traits. However, it is instructive to re-emphasize the point that high proportion of phenotypic variation explainable by population of origin cannot alone provide evidence for divergent natural selection, because such a pattern could be generated by sufficiently strong random drift too. Similarly, even though a great proportion of phenotypic variation in many traits can be explained by habitat, this can in principle result from the fact that populations in similar environments may have a shared evolutionary history. Thus, it is crucial that both *S* and *H* tests account for the influence of evolutionary history by viewing the distribution generated by neutral genetic drift as a null model.

To sum up, we have devised an improved approach to detect signatures of natural selection in quantitative traits, and demonstrated it to be suitable and powerful also in situations where the number of study populations and sampled individuals are low. We have illustrated the virtues of this approach by using empirical data on differentiation of quantitative traits among marine and pond populations of nine-spined sticklebacks—a study system where detection of signatures of natural selection with traditional methods is challenged by a high level of random drift in neutral marker genes. The method developed in this study should provide a useful framework to test for adaptive nature of population

differentiation in many kinds of study systems, in particular ones with a small number of local populations or a high rate of random genetic drift.

ACKNOWLEDGMENTS

The authors thank A. Gonda for her help in collecting, rearing, and measuring the fish, as well as the numerous people who helped us in locating and obtaining fish used in this study. The authors also thank T. Shikano for access to the earlier published microsatellite data and two anonymous referees for constructive criticism which improved this manuscript. Our work was funded by Academy of Finland (Grants 200940, 108601, and 118673 to JM and 250444 to OO), and the European Research Council (ERC Starting Grant 205905 to OO).

LITERATURE CITED

Ab Ghani, N. I., G. Herczeg, and J. Merilä. 2012. Body size divergence in nine-spined sticklebacks: disentangling additive genetic and maternal effects. Biol. J. Linn. Soc. 107:521–528.

Ab Ghani, N. I., G. Herczeg, T. Leinonen, and J. Merilä. 2013. Evidence for genetic differentiation in timing of maturation among nine-spined stickleback populations. J. Evol. Biol. 26:775–782.

Blanquart, F., S. Gandon, and S. L. Nuismer. 2012. The effects of migration and drift on local adaptation to a heterogeneous environment. J. Evol. Biol. 25:1351–1363.

Brommer, J. E. 2011. Whither *P*(st)? The approximation of Q(st) by P(st) in evolutionary and conservation biology. J. Evol. Biol. 24:1160–1168.

Bruneaux, M., S. E. Johnston, G. Herczeg, J. Merilä, C. R. Primmer, and A. Vasemägi. 2013. Molecular evolutionary and population genomic analysis of the nine-spined stickleback using a modified restriction-site-associated DNA tag approach. Mol. Ecol. 22:565–582.

Chapuis, E., G. Martin, and J. Goudet. 2008. Effects of selection and drift on G matrix evolution in a heterogeneous environment: a multivariate *Q*(st) − *F*(st) test with the freshwater snail *Galba truncatula*. Genetics 180:2151–2161.

Coop, G., D. Witonsky, A. Di Rienzo, and J. K. Pritchard. 2010. Using environmental correlations to identify loci underlying local adaptation. Genetics 185:1411–1423.

De Mita, S., A.-C. Thuillet, L. Gay, N. Ahmadi, S. Manel, J. Ronfort, and Y. Vigouroux. 2013. Detecting selection along environmental gradients: analysis of eight methods and their effectiveness for outbreeding and selfing populations. Mol. Ecol. 22:1383–1399.

DeFaveri, J., T. Shikano, N. I. Ab Ghani, and J. Merilä. 2012. Contrasting population structures in two sympatric fishes in the Baltic Sea basin. Marine Biol. 159:1659–1672.

DeWoody, J. A., and J. C. Avise. 2000. Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. J. Fish Biol. 56:461–473.

Edelaar, P., P. Burraco, and I. Gomez-Mestre. 2011. Comparison between *Q*(st) and *F*(st)—how wrong have we been? Mol. Ecol. 20:4830–4839.

Egea, R., S. Casillas, and A. Barbadilla. 2008. Standard and generalized McDonald–Kreitman test: a website to detect selection by comparing different classes of DNA sites. Nucleic Acids Res. 36:W157–W162.

Foll, M., and O. Gaggiotti. 2008. A genome-scan method to identify selected loci appropriate for both dominant and codominant markers: a Bayesian perspective. Genetics 180:977–993.

Gienapp, P., C. Teplitsky, J. S. Alho, J. A. Mills, and J. Merilä. 2008. Climate change and evolution: disentangling environmental and genetic responses. Mol. Ecol. 17:167–178.

Gonda, A., G. Herczeg, and J. Merilä. 2009a. Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? J Evol. Biol. 22:1721–1726.

———. 2009b. Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain. Proc. R. Soc. B 276:2085–2092.

Gonda, A., K. Välimäki, G. Herczeg, and J. Merilä. 2012. Brain development and predation: plastic responses depend on evolutionary history. Biol. Letters 8:249–252.

Hendry, A. P. 2002. *Q*(st) not equal to *F*(st)? Trends Ecol. Evol. 17:502–502.

Herczeg, G., and K. Välimäki. 2011. Intraspecific variation in behaviour: effects of evolutionary history, ontogenetic experience and sex. J. Evol. Biol. 24:2434–2444.

Herczeg, G., A. Gonda, and J. Merilä. 2009a. Evolution of gigantism in nine-spined sticklebacks. Evolution 63:3190–3200.

———. 2009b. Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). J. Evol. Biol. 22:544–552.

———. 2009c. The social cost of shoaling covaries with predation risk in nine-spined stickleback, *Pungitius pungitius*, populations. Anim. Behav. 77:575–580.

Herczeg, G., M. T. Turtiainen, and J. Merilä. 2010. Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. Biol. J. Linn. Soc. 101: 403–416.

Herczeg, G., A. Gonda, A. Kuparinen, and J. Merilä. 2012. Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? Evol. Ecol. 26:109–122.

Herczeg, G., N. I. Ab Ghani, and J. Merilä. 2013. Evolution of stickleback feeding behaviour: genetics of population divergence at different ontogenetic stages. J. Evol. Biol. 26:955–962.

Hereford, J. 2009. A quantitative survey of local adaptation and fitness trade-offs. Am. Nat. 173:579–588.

Karhunen, M., and O. Ovaskainen. 2012. Estimating population-level coancestry coefficients by an admixture F model. Genetics 192: 609–617.

Karhunen, M., O. Ovaskainen, T. Leinonen, and J. Merilä. 2013. driftsel: an R package for detecting signals of natural selection in quantitative traits. Mol. Ecol. Res. 13:746–754.

Kawecki, T. J., and D. Ebert. 2004. Conceptual issues in local adaptation. Ecol. Lett. 7:1225–1241.

Kronholm, I., O. Loudet, and J. de Meaux. 2010. Influence of mutation rate on estimators of genetic differentiation—lessons from *Arabidopsis thaliana*. BMC Genet. 11:33–46.

Kuparinen, A., and J. Merilä. 2008. The role of fisheries-induced evolution. Science 320:47–48.

Langerhans, R. B., and T. J. DeWitt. 2004. Shared and unique features of evolutionary diversification. Am. Nat. 164:335–349.

Le Corré, V., and A. Kremer. 2012. The genetic differentiation at quantitative trait loci under local adaptation. Mol. Ecol. 21:1548–1566.

Leinonen, T., R. J. S. McCairns, R. B. O'Hara, and J. Merilä. 2013. *Q*(st) − *F*(st) comparisons: evolutionary and ecological insights from genomic heterogeneity. Nat. Rev. Genet. 14:179–190.

Leinonen, T., R. B. O'Hara, J. M. Cano, and J. Merilä. 2008. Comparative studies of quantitative trait and neutral marker divergence: a meta-analysis. J. Evol. Biol. 21:1–17.

Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27:209–220.

McKay, J. K., and R. G. Latta. 2002. Adaptive population divergence: markers, QTL and traits. Trends Ecol. Evol. 17:285–291.

Merilä, J. 2013. Nine-spined stickleback (*Pungitius pungitius*): an emerging model for evolutionary biology research. Ann. NY Acad. Sci. 1289:18–35.

Merilä J., and Crnokrak P. 2001. Comparison of genetic differentiation at marker loci and quantitative traits. J. Evol. Biol. 14, 892–903.

Narum, S. R., and J. E. Hess. 2011. Comparison of *F*(st) outlier tests for SNP loci under selection. Mol. Ecol. Res. 11:184–194.

Nosil, P., D. J. Funk, and D. Ortiz-Barrientos. 2009. Divergent selection and heterogeneous genomic divergence. Mol. Ecol. 18:375–402.

O'Hara, R. B., and J. Merilä. 2005. Bias and precision in *Q*(st) estimates: problems and some solutions. Genetics 171:1331–1339.

Ovaskainen, O., M. Karhunen, C. Z. Zheng, J. M. C. Arias, and J. Merilä. 2011. A new method to uncover signatures of divergent and stabilizing selection in quantitative traits. Genetics 189:621–729.

Pujol, B., A. J. Wilson, R. I. C. Ross, and J. R. Pannell. 2008. Are *Q*(st) − *F*(st) comparisons for natural populations meaningful? Mol. Ecol. 17:4782–4785.

Rhone, B., R. Vitalis, I. Goldringer, and I. Bonnin. 2010. Evolution of flowering time in experimental wheat populations: a comprehensive approach to detect genetic signatures of natural selection. Evolution 64:2110–2125.

Rohlf, F. J. 2007. Department of Ecology and Evolution, State University of New York, Stony Brook, NY. Available at tpsRelw. http://life.bio.sunysb.edu/ee/rohlf/software.html.

———. 2008. Available at tpsRelw. http://life.bio.sunysb.edu/ee/rohlf/software.html, tpsDig. http://life.bio.sunysb.edu/ee/rohlf/software.html, Department of Ecology and Evolution, State University of New York, Stony Brook, NY.

Rousset, F. 2002. Inbreeding and relatedness coefficients: what do they measure? Heredity 88:371–380.

Schluter, D. 2000. Ecological character displacement in adaptive radiation. Am. Nat. 156:S4–S16.

Shikano, T., Y. Shimada, G. Herczeg, and J. Merilä. 2010. History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. Mol. Ecol. 19:1147–1161.

Shimada, Y., T. Shikano, A. Kuparinen, A. Gonda, T. Leinonen, and J. Merilä. 2011. Quantitative genetics of body size and timing of maturation in two nine-spined stickleback (*Pungitius pungitius*) populations. PLoS One 6:e28859.

Spitze, K. 1993. Population structure in *Daphnia obtusa*: quantitative genetic and allozymic variation. Genetics 135:367–374.

Storz, J. F. 2005. Using genome scans of DNA polymorphism to infer adaptive population divergence. Mol. Ecol. 14:671–688.

Trokovic, N., G. Herczeg, R. J. S. McCairns, N. I. Ab Ghani, and J. Merilä. 2011. Intraspecific divergence in the lateral line system in the nine-spined stickleback (*Pungitius pungitius*). J. Evol. Biol. 24: 1546–1558.

Trokovic, N., G. Herczeg, N. I. Ab Ghani, T. Shikano, and J. Merilä. 2012. High levels of fluctuating asymmetry in isolated stickleback populations. BMC Evol. Biol. 12:115–123.

Tsumura, Y., K. Uchiyama, Y. Moriguchi, S. Ueno, and T. Ihara-Ujino. 2012. Genome scanning for detecting adaptive genes along environmental gradients in the Japanese conifer, *Cryptomeria japonica*. Heredity 109:349–360.

Ward, R. D., M. Woodwark, and D. O. Skibinsky. 1994. A comparison of genetic diversity in marine, freshwater and anadromous fishes. J. Fish Biol. 44:213–232.

Waser, W., T. P. Sahoo, G. Herczeg, J. Merilä, and M. Nikinmaa. 2010. Physiological differentiation among nine-spined stickleback populations: effects of copper exposure. Aquat. Toxicol. 98:188–195.

Whitlock, M. C., and F. Guillaume. 2009. Testing for spatially divergent selection: comparing *Q*(st) to *F*(st). Genetics 183:1055–1063.

Whitlock, M. C., and K. J. Gilbert. 2012. *Q*(st) in a hierarchically structured population. Mol. Ecol. Res. 12:481–483.

Wootton, R. J. 1984. A functional biology of sticklebacks. Croom Helm, Lond.

Välimäki, K., G. Herczeg, and J. Merilä. 2012. Morphological anti-predator defences in the nine-spined stickleback: constitutive, induced or both? Biol. J. Linn. Soc. 107:854–866.

Associate Editor: R. Fuller

dc_892_14

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Appendix A. Including binary traits.
Figure A1. The statistical model of driftsel extended to binary variables.

Ann. Zool. Fennici 49: 123–128 ISSN 0003-455X (print), ISSN 1797-2450 (online)
Helsinki 20 April 2012

Isolation and characterization of 13 new nine-spined stickleback, *Pungitius pungitius*, microsatellites located nearby candidate genes for behavioural variation

Veronika N. Laine[1,*], Craig R. Primmer[1], Gabor Herczeg[2], Juha Merilä[2] & Takahito Shikano[2]

[1)] *Division of Genetics and Physiology, Department of Biology, FI-20014 University of Turku, Finland (corresponding author's e-mail: veronika.laine@utu.Â)*
[2)] *Ecological Genetics Research Unit, Department of Biosciences, P.O. Box 65, FI-00014 University of Helsinki, Finland*

Received 9 Aug. 2011, Ânal version received 18 Nov. 2011, accepted 22 Nov. 2011

Laine, V. N., Primmer, C. R., Herczeg, G., Merilä, J. & Shikano, T. 2012: Isolation and characterization of 13 new nine-spined stickleback, *Pungitius pungitius*, microsatellites located nearby candidate genes for behavioural variation. — *Ann. Zool. Fennici* 49: 123–128.

Nine-spined stickleback (*Pungitius pungitius*) is a good model species for studying personality traits such as boldness and aggression as large variation in these behaviours has been observed in populations from different habitats. Here we used genomic information from three-spined sticklebacks to identify and characterise 13 new nine-spined stickleback microsatellite loci which are located close to candidate genes for behaviour. All loci were polymorphic with 3–31 alleles per locus and expected heterozygosity varied from 0 to 0.97 and observed heterozygosity from 0 to 1. These markers should provide a useful resource for better understanding the genetic basis of behaviour in stickleback Âshes.

Individual and population-level differences in behavioural traits such as aggression and boldness are suggested to be widespread in the animal kingdom and to have important Âtness consequences (e.g. Sih *et al.* 2004a, 2004b, Reale *et al.* 2007). Despite their potential ecological and evolutionary signiÂcance, the underlying genetic bases of such behaviours have rarely been studied in free-living populations. However, recent studies have demonstrated how knowledge of genes that have been demonstrated to be associated with personality traits in "model" organisms (including humans) can be utilized for studying the genetic basis of behavioural variation in wild animal populations through the use of a 'candidate gene approach' (Fidler *et al.* 2007).

Nine-spined sticklebacks (*Pungitius pungitius*) are a good model species for behavioural ecology studies because Âsh from different habitats in Fennoscandia exhibit differing behavioural averages, with Âsh from isolated pond populations being quicker in feeding, bolder and more aggressive than individuals from marine environments (Herczeg *et al.* 2009, Herczeg and Välimäki 2011). In addition to these features, nine-spined sticklebacks are amenable to controlled experiments and there is access to detailed genome sequence information (http://www.ensembl.org/Gasterosteus_aculeatus/Info/Index) from a related species, the three-spined stickleback (*Gasterosteus aculeatus*). These two lineages have diverged more than 10 million years ago which is equivalent to 5–10 millions of generations (Baker 1994, Bell 1994).

Earlier studies have demonstrated that molecular markers located within or nearby target candidate genes can be a useful resource for the identiÂcation of genes associated with adaptive phenotypic divergence especially when there is no other genomic resources available (Shikano *et al.* 2010a, Tonteri *et al.* 2010). In addition, markers closely linked to functionally important genes are useful in construction of comparative genetic maps, in which they can be used as comparative anchor-tagged sequence loci (Lyons *et al.* 1997). Here we report 13 new polymorphic microsatellite loci for nine-spined sticklebacks. These loci are located near genes that have earlier been shown to be associated with behavioural variation in other species.

Microsatellite markers were designed using a candidate-gene approach (Fitzpatrick *et al.* 2005, Shikano *et al.* 2010b). Genes earlier shown to be associated with aggression and boldness were identiÂed using data available from literature on human, mouse and domestic-animal genetics (Table 1). Altogether 51 candidate genes associated with aggression and boldness variation were identiÂed and homologues were then identiÂed by using the gene name or the homologue sequence for all these genes in the three-spined stickleback genome obtained from Ensembl (Genebuild January 2009, database version 56.1j/57.1j).

Candidate genes and genomic regions closely linked to the genes (< 10 kb) were screened for microsatellite repeats using the Phobos 3.3.11 software (http://www.ruhr-uni-bochum.de/spezzoo/cm/cm_phobos.htm) included in Geneious ver. 5.4 (http://www.geneious.com/) where repeat unit length was set from one to four. Homologous gene sequences from additional teleost Âsh species such as medaka (*Oryzias latipes*), fugu (*Takifugu rubripes*) and zebraÂsh (*Danio rerio*) were aligned with three-spined stickleback gene sequences in order to identify conserved sequence regions Âanking the microsatellite repeat region. Primers were designed manually so that GC content was 45%–60%, the primer melting temperature was approximately 60 °C and the length of the primers was 20–26 bp. The aim was for the primers to amplify the microsatellite region with a fragment size of approximately 800 bp. In the three-spined stickleback genome sequence, suitable microsatellite regions were found in a total of 30 genes and in some cases multiple primers were designed for the microsatellite region, or for different microsatellites nearby the same gene. Genomic DNA from nine-spined stickleback individuals from Pyöreälampi, Finland (66°15´N, 29°26´E) were used for testing of primers by amplifying and sequencing the target regions while DNA from one three-spined stickleback individual caught in the Baltic Sea near Helsinki (60°10´N, 25°00´E) was used as a positive control. DNA was extracted from Ân samples by using a modiÂed salt extraction protocol of Aljnabi and Martinez (1997).

PCR reactions were carried out in a 20 µl reaction volume consisting of 1 PCR buffer (Bioline, London, UK), 1.5 mM $MgCl_2$, 0.25 mM dNTP (Finnzymes, Espoo, Finland), 0.1 U BIOTAQ DNA polymerase (Bioline, London, UK), 10 pmol of each primer and approx. 30 ng of genomic DNA. The initial touchdown PCR proÂle was as follows: initial degeneration step at 95 °C for 3 minutes (min), followed by 20 cycles of denaturation for 30 seconds (s) at 95 °C, annealing starting at 60 °C for 30 s and dropping by 0.5 °C per cycle and extension at 72 °C for 1 min, followed by 20 cycles of 30 s denaturation at 94 °C and 30 s annealing at 50 °C and extension at 72 °C for 1 min with a Ânal extension at 72 °C for 6 min. The PCR product proÂles for speciÂc loci were then optimised by either increasing or decreasing the annealing temperature. The success of PCR ampliÂcation and the size of the amplicons were determined by electrophoresis on 1.5% agarose gel with a DNA ladder (GeneRuler™ DNA Ladder Mix, Fermentas, Helsinki, Finland). PCR products deemed suitable for sequencing were prepared using exonuclease I (Fermentas, Helsinki, Finland) and shrimp alkaline phosphatase (Fermentas, Helsinki, Finland) and sequenced directly in both forward and reverse directions with the primers used in the PCRs. The sequencing reactions were performed in 10 µl volumes using a

Table 1. Microsatellite loci for the nine-spined stickleback, including adjacent gene name (abbreviation) and a citation where association with a personality trait has been reported, Genbank accession number, primer sequences, the dye used, repeat motif, total number of alleles (Total A), size range (base-pairs) and amplification quality rating.

Locus	Gene name	Prior behaviour association	Genbank	Primer sequence[1] (5′–3′)	Dye	Repeat	Total A	Size (bp)	Quality rating[2]
Ppbig1	Androgen receptor (*AR*)	Dominance in fish (Burmeister *et al.* 2007)	JQ012804	F- AGTGAGGATTCGGAGGGCA R- GTTTCTGACGAGGAGAGGCACA	NED	$(AC)_{12}$	5	172–182	little stutter
Ppbig2	Cholecystokinin B receptor (*CCK-B*)	Anxiety in rats (Wang *et al.* 2005)	JQ012805	F- CCCATCCAACGGCAGAGCA R- GTTTGGTGCGGCTGCCCAGATTT	FAM	$(AT)_{11}$	5	279–293	little stutter
Ppbig3	Corticotropin-releasing hormone (*CRH*)	Aggression in fish (Filby *et al.* 2010)	JQ012806	F- TCCCTGCTGCTGCATTGTTGG R- GTTTCGTTAGTCGTCAACCGGCTGT	VIC	$(C)_9$	5	276–282	moderate stutter
Ppbig4	Dopamine transporter (*DAT1*)	Aggression in humans (Chen *et al.* 2005)	JQ012807	F- ACCTGGATCCTCCGTTTGGGC R- GTTTCCATCTGTACCCTGGCT	VIC	$(C)_7$,ACA(C)$_4$	3	345–348	no stutter
Ppbig5	Dopamine receptor D_1 (*DRD1*)	Aggression in dogs (Vage *et al.* 2010)	JQ012808	F- CACGAACACAGGAGGACGGA R- GTTTCGCATCTGTACCCTGGCAT	PeT	$(AC)_{43}$	28	280–384	little stutter
Ppbig6	Dopamine receptor D_4 (*DRD4*)	Novelty seeking in birds (Fidler *et al.* 2007)	JQ012809	F- CTGCGGACGACCTTGACACC R- GTTTCGCCCGGAAGCATTCTCC	FAM	$(A)_{23}$	15	335–354	moderate stutter
Ppbig7	estrogen receptor beta (*ESR2*)	Anxiety in rats (Lund *et al.* 2005)	JQ012810	F- GCCGCCGGAGATCCTCCTCA R- GTTTGTGTCTGGCGTCGCAT CGG	NED	$(A)_7$	4	281–301	no stutter
Ppbig8	5 hydroxytryptamine receptor 3B (*HTR3B*)	Anticocaïlly in humans (Ducci *et al.* 2009)	JQ012811	F- GGACTGCTGATCCACACAAACAA R- GTTTCTCCACAGCGCCAGGTCTA	VIC	$(AC)_{11}$	10	148–170	no stutter
Ppbig9	Monoamine oxidase A (*MAOA*)	Aggression in humans (Brunner *et al.* 1993)	JQ012812	F- TAAACCGCCTGATTGTGTGC R- GTTTAAGAAACGGGAGGTGAGG	FAM	$(C)_{15}$	6	94–107	moderate stutter
Ppbig10	Progesterone receptor (*PGR*)	Panic disorder in humans (Ho et a. 2004)	JQ012813	F- TCCGGATGACTCACTCTTCAC R- GTTTCAAATTCTCCTCTCGGTGTACCA	PeT	$(AC)_{16}$	31	139–255	little stutter
Ppbig11	Protein kinase G	Food related behaviour (Osborne *et al.* 1997)	JQ012814	F- CGGCTGTCTATGGCGCATCT R- GTTTCGTCCCGCTCTTTGACCCTT	NED	$(AC)_{18}$	13	395–419	little stutter
Ppbig12	excitatory amino acid transporter 2 (*SLC1A2*)	Activity in dogs (Takeuchi *et al.* 2009)	JQ012815	F- TCCCAGAGAACTTGGTCAGGCT R- GTTTGCCATCACTTGACGATTGTATGTC	FAM	$(C)_{16}$	9	152–162	little stutter
Ppbig13	Serotonin transporter (*SLC6A4*)	Aggression in rats (Holmes *et al.* 2003)	JQ012816	F- CAGGACTGGGTGCTGTGGC R- GTTTACCAGGGTGCAATTGTTGTGAGC	NED	$(AAC)_6$	3	104–110	no stutter

[1] the GTTT 'tail' (underlined) was added to the 5′ end of each non-labelled primer to enhance 3′OH A addition to the forward strand (Brownstein *et al.* 1996).
[2] see Primmer & Merilä 2002.

BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Espoo, Finland) according to manufacturer's instructions. Obtained sequencing products were cleaned with a Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore, Espoo, Finland). Sequencing products were visualised with an ABI 3130xl DNA Analyzer (Applied Biosystems, Espoo, Finland).

Sequences were aligned and edited in the Geneious analysis package using both forward and reverse sequences. BLASTN searches against the three-spined stickleback genome were used to ensure that the sequences obtained were homologous to the expected regions. In all cases, BLASTN also revealed similarity with homologous gene regions in more distantly related fish species such as zebrafish and therefore it is likely that the markers are linked to similar gene regions in nine-spined sticklebacks. Primer3 (Rozen 2000; source code available from http://fokker.wi.mit.edu/primer3/) included in Geneious was used to design primers generating a product of 100–500 bp. Fragment lengths were selected so as to optimise the number of markers with non-overlapping size ranges. Subsequent PCR optimisation and polymorphism detection was conducted using genomic DNA from eight individuals from two different populations. The final panel of 13 polymorphic markers was then optimised to enable amplification in a single multiplex PCR reaction that included 1 Qiagen Multiplex PCR Master Mix, 0.08 µM fluorescent labelled forward primer, 0.08 µM reverse primer (for *ppbig11* the concentration for forward and reverse was 0.1 µM), approx. 30 ng of genomic DNA, and dH_2O to a final volume of 7 µl. The thermal cycling program for the multiplex was: 95 °C for 15 min; 35 cycles of 94 °C for 30 s, 61 °C for 1 min 30 s, 72 °C for 1 min 30 sec, followed by 60 °C for 30 min. PCR products were subsequently diluted by adding 5 µl of water to the PCR wells and then diluting 1:200 and electrophoresed using an ABI3130xl Genetic Analyzer with GeneScan-600 LIZ size standard (Applied Biosystems, Foster City, CA). Genotypes were scored using GeneMapper 4.0 (Applied Biosystems, Foster City, CA). Genetic diversity levels were determined by genotyping 10 putatively unrelated individuals from each of four populations: two sampled from small ponds, Pyöreälampi (PYÖ) and two sampled from the marine environment, Helsinki (HKI) and Levin Navolok, White Sea (66°18′N, 33°25′E, LEV). A map indicating the population locations can be found in Herczeg *et al.* (2009).

Genetic diversity indices were calculated using the MICROSATELLITE TOOLKIT EXCEL add-in (Park 2001) and tests for Hardy-Weinberg and linkage equilibrium were conducted using GENEPOP ver. 4.0 (Raymond & Rousset 1995, Rousset 2008). Sequences for all loci reported have been submitted to Genbank (Accession numbers JQ012804-JQ012816).

Mono-, di- or trinucleotide repeat sequences were identified in 22 of the 30 regions amplified and one to three primer pairs were designed in efforts to amplify these 22 loci from nine-spined sticklebacks genomic DNA. Fourteen of the 22 loci revealed length polymorphisms in nine-spined stickleback's DNA. However one of these loci proved to PCR-amplify unreliably and a total of 13 loci were optimised to be PCR amplified in a single PCR reaction (Table 1).

After Bonferroni correction (Rice 1989), all loci conformed to Hardy-Weinberg expectations in every population. None of the 13 loci was in linkage equilibrium with each other. Across all four populations, the number of alleles observed per locus ranged from 3 to 31. At the population level, observed heterozygosities ranged from 0 to 0.90 (6 loci > 0.5) in BYN, from 0.50 to 1.0 (13 loci > 0.5) in HKI, from 0.20 to 1.0 (11 loci > 0.5) in LEV and from 0 to 0.60 (1 locus > 0.5) in PYÖ (Table 2). The lower levels of observed heterozygosities in populations from pond habitats (BYN, PYÖ) when compared with those from marine habitats (HKI, LEV) were also found in earlier studies (Shikano *et al.* 2010c). Some cross-species testing was conducted using three-spined stickleback genomic DNA and at least two of the markers (*Ppbig1* and *Ppbig5*) revealed polymorphisms (data not shown). These markers add to the anonymous markers and are linked to physiologically important genes currently available for nine-spined sticklebacks (Koizumi *et al.* 2007, Meguro *et al.* 2009, Shapiro *et al.* 2009, Shikano *et al.* 2010b). Overall, these markers should provide a useful resource for better understanding the genetic basis of behaviour in sticklebacks.

Table 2. Population speciÀc diversity indices for four nine-spined stickleback populations (n = 10 per population): number of alleles (A), observed heterozygosity (HO), expected heterozygosity (Hc), P-value for deviation from Hardy-Weinberg equilibrium (H-W) (prior to Bonferroni correction).

	BYN				HKI				LcV				PYÖ			
Locus	A	HO	Hc	H-W*	A	HO	Hc	H-W	A	HO	Hc	H-W	A	HO	Hc	H-W*
Ppbig1	2	0.1	0.27	0.16	3	0.5	0.58	0.45	4	0.5	0.65	0.01	2	0.4	0.34	1
Ppbig2	2	0.2	0.19	1	4	0.6	0.77	0.9	3	0.4	0.64	0.21	2	0.2	0.19	1
Ppbig3	2	0.6	0.51	1	5	0.9	0.77	0.51	2	0.5	0.39	1	1	0	0	–
Ppbig4	1	0	0	–	3	0.7	0.56	0.74	2	0.2	0.19	1	1	0	0	–
Ppbig5	4	0.6	0.68	0.74	14	1	0.95	1	15	1	0.97	1	3	0.4	0.56	0.31
Ppbig6	2	0.6	0.44	0.48	12	1	0.95	1	9	1	0.83	0.67	3	0.4	0.54	0.68
Ppbig7	3	0.5	0.59	0.24	4	0.6	0.6	0.84	3	0.9	0.69	0.51	1	0	0	–
Ppbig8	1	0	0	–	7	0.9	0.82	0.9	7	0.6	0.77	0.23	2	0.3	0.27	1
Ppbig9	2	0.3	0.27	1	6	0.9	0.8	0.8	3	1	0.57	0.01	2	0.2	0.19	1
Ppbig10	6	0.9	0.78	0.99	15	1	0.97	1	15	0.9	0.96	0.37	5	0.6	0.62	0.57
Ppbig11	3	0.6	0.53	1	6	0.7	0.66	0.62	9	0.7	0.79	0.12	2	0.1	0.1	–
Ppbig12	1	0	0	–	6	0.7	0.62	0.82	7	0.9	0.85	0.95	1	0	0	–
Ppbig13	1	0	0	–	3	0.6	0.57	0.03	3	0.7	0.65	0.11	1	0	0	–
Average	2	0.34	0.33	0.73	7	0.78	0.74	0.74	6	0.72	0.69	0.48	2	0.20	0.22	0.79

* results indicated with '–' are either monomorphic or loci with very low heterozygosity, hence the H-W value could not be calculated.

Acknowledgements

We thank Heidi Viitaniemi and Erica Leder for supplying three-spined stickleback samples. VL was Anancially supported by the Biological Interactions Graduate School. This study was funded by Centre of Excellence in Evolutionary Genetics and Physiology and Academy of Finland.

References

Aljnabi, S. M. & Martinez, I. 1997: Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. — *Nucleic Acids Research* 25: 4629–4693.

Baker, J.A. 1994: Life history variation in female threespine stickleback. — In: Bell, M. A. & Foster, S. A. (eds.), *The evolutionary biology of the threespine stickleback*: 144–187. Oxford University Press Oxford.

Bell, M. A. 1994: Palaeobiology and evolution of threespine stickleback. — In: Bell, M. A. & Foster, S. A. (eds.), *The evolutionary biology of the threespine stickleback*: 438–471. Oxford University Press Oxford.

Brownstein, M. J., Carpten, J. D. & Smith, J. R. 1996: Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modiÀcations that facilitate genotyping. — *BioTech* 20: 1004–1010.

Brunner, H. G., Nelen, M., BreakeAeld, X. O., Ropers, H. H. & van Oost, B. A. 1993: Abnormal behavior associated with a point mutation in the structural gene for monoamine oxidase A. — *Science* 262: 578–580.

Burmeister, S. S., Kailasanath, V. & Fernald, R. D. 2007: Social dominance regulates androgen and estrogen receptor gene expression. — *Hormones and Behavior* 51: 164–170.

Chen, T. J. H., Blum, K., Mathews, D., Fisher, L., Schnautz, N., Braverman, E. R., SchoolAeld, J., Downs, B. W. & Comings, D. E. 2005: Are dopaminergic genes involved in a predisposition to pathological aggression? Hypothesizing the importance of "super normal controls" in psychiatricgenetic research of complex behavioral disorders. — *Medical Hypotheses* 65: 703–707.

Ducci, F., Enoch, M. A., Yuan, Q., Shen, P. H., White, K. V., Hodgkinson, C., Albaugh, B., Virkkunen, M. & Goldman, D. 2009: *HTR3B* is associated with alcoholism with antisocial behavior and alpha EEG power — an intermediate phenotype for alcoholism and co-morbid behaviors. — *Alcohol* 43: 73–84.

Fidler, A. E., van Oers, K., Drent, P. J., Kuhn, S., Mueller, J. C. & Kempenaers, B. 2007: *Drd4* gene polymorphisms are associated with personality variation in a passerine bird. — *Proceedings of the Royal Society B* 274: 1685–1691.

Filby, A. L., Paull, G. C., Hickmore, T. F. A. & Tyler, C. R. 2010: Unravelling the neurophysiological basis of aggression in a Ash model. — *BMC Genomics* 11: 498.

Fitzpatrick, M. J., Ben-Shahar, Y., Smid, H. M., Vet, L. E. M., Robinson, G. E. & Sokolowski, M. B. 2005: Candidate genes for behavioural ecology. — *Trends in Ecology and Evolution* 20: 96–104.

Herczeg, G. & Välimäki, K. 2011: IntraspeciÀc variation in behaviour: effects of evolutionary history, ontogenetic experience and sex. — *Journal of Evolutionary Biology* 34: 2434–2444.

Herczeg, G., Gonda, A. & Merilä, J. 2009: Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). — *Journal of Evolutionary Biology* 22: 544–552.

Ho, H. P., Westberg, L., Annerbrink, K., Olsson, M., Melke, J., Nilsson, S., Baghaeie, F., Rosmonde, R., Holme, G., Björntorpe, P., Anderschf, S., Allgulanderg, C. & Eriksson, E. 2004: Association between a functional polymorphism in the progesterone receptor gene and panic disorder in women. — *Psychoneuroendocrinology* 29: 1138–1141.

Holmes, A., Murphy, D. L. & Crawley, J. N. 2003: Abnormal behavioral phenotypes of serotonin transporter knockout mice: parallels with human anxiety and depression. — *Biological Psychiatry* 54: 953–959.

Koizumi, N., Jinguji, H., Takahashi, H., Higuchi, M., Takata, K., Minezawa, M., Takemura, T. & Mori, A. 2007: Isolation and characterization of polymorphic microsatellite DNA markers in the Omono type of ninespine stickleback, genus *Pungitius*. — *Molecular Ecology Notes* 7: 1315–1318.

Lund, T. D., Rovis, T., Chung, W. C. J. & Handa, R. J. 2005: Novel actions of estrogen receptor-β on anxiety-related behaviors. — *Endocrinology* 146: 797–807.

Lyons, L. A., Laughlin, T. F., Copeland, N. G., Jenkins, N. A., Womack, J. E. & O'Brien, S. J. 1997: Comparative anchor tagged sequences (CATS) for integrative mapping of mammalian genomes. — *Nature Genetics* 15: 47–56.

Meguro, Y., Takahashi, H., Takeshima, H., Nishida, M. & Goto, A. 2009: Isolation and characterization of 13 microsatellite loci in the nine-spined stickleback (*Pungitius pungitius*) and cross-species ampliÀcation in 5 stickleback species (family Gasterosteidae). — *Conservation Genetics Resources* 1: 31–34.

Osborne, K. A., Robichon, A., Burgess, E., Butland, S., Shaw, R. A., Coulthard, A., Pereira, H. S., Greenspan, R. J. & Sokolowski, M. B. 1997: Natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. — *Science* 277: 834–836.

Park, S. D. E. 2001: *Trypanotolerance in West African cattle and the population genetic effects of selection*. — Ph.D. thesis, University of Dublin.

Primmer, C. R. & Merilä, J. 2002: A low rate of cross-species microsatellite ampliÀcation success in ranid frogs. — *Conservation Genetics* 3: 445–449.

Raymond, M. & Rousset, F. 1995: GENEPOP (version 1.2), population genetics software for exact tests and ecumenicism. — *Journal of Heredity* 86: 248–249.

Réale, D., Reader, S. M., Sol, D., McDougall, P. T. & Dingemanse, N. J. 2007: Integrating animal temperament within ecology and evolution. — *Biological Reviews* 82: 291–318.

Rice, W. R. 1989: Analysing tables of statistical tests. — *Evolution* 43: 223–225.

Rousset, F. 2008: Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. — *Molecular Ecology Notes* 8:103–106.

Rozen, S. & Skaletsky, H. J. 2000: Primer3 on the WWW for general users and for biologist programmers. — In: Krawetz, S. & Misener, S. (eds.), *Bioinformatics: Methods and protocols (Methods in molecular biology)*: 365–386. Humana Press, Totowa, NJ.

Shapiro, M. D., Summers, B. R., Balabhadra, S., Aldenhoven, J. T., Miller, A. L., Cunningham, C. B., Bell, M. A. & Kingsley, D. M. 2009: The genetic architecture of skeletal convergence and sex determination in ninespine sticklebacks. — *Current Biology* 19: 1140–1145.

Shikano, T., Ramadevi, J. & Merilä, J. 2010a: IdentiÀcation of local- and habitat-dependent selection: scanning functionally important genes in nine-spined sticklebacks (*Pungitius pungitius*). — *Molecular Biology and Evolution* 27: 2775–2789.

Shikano, T., Ramadevi, J., Shimada, Y. & Merilä, J. 2010b: Utility of sequenced genomes for microsatellite marker development in non-model organisms: a case study of functionally important genes in nine-spined sticklebacks (*Pungitius pungitius*). — *BMC Genomics* 11: 334.

Shikano, T., Shimada, Y., Herczeg, G. & Merilä, J. 2010c: History *vs.* habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. — *Molecular Ecology* 19: 1147–1161.

Sih, A., Bell, A. & Johnson, C. 2004a: Behavioural syndromes: an ecological and evolutionary overview. — *Trends in Ecology and Evolution* 19: 372–378.

Sih, A., Bell, A. M., Johnson, J. C. & Ziemba, R. E. 2004b: Behavioral syndromes: an integrative overview. — *Quarterly Review of Biology* 79: 241–277.

Takeuchi, Y., Hashizume, C., Arata, S., Inoue-Murayama, M., Maki, T., Hart, B. L. & Mor, Y. 2009: An approach to canine behavioural genetics employing guide dogs for the blind. — *Animal Genetics* 40: 217–224.

Tonteri, A., Vasemägi, A., Lumme, J. & Primmer, C. R. 2010: Beyond MHC: signals of elevated election pressure on Atlantic salmon (*Salmo salar*) immune-relevant loci. — *Molecular Ecology* 19: 1273–1282.

Väge, J., Wade, C., Biagi, T., Fatjó, J., Amat, M., Lindblad-Toh, K. & Lingaas, F. 2010: Association of dopamine- and serotonin-related genes with canine aggression. — *Genes, Brain and Behavior* 9: 372–378.

Wang, H., Wong, P. T.-H., Spiess, J. & Zhu Y. Z. 2005: Cholecystokinin-2 (CCK2) receptor-mediated anxiety-like behaviors in rats. — *Neuroscience & Biobehavioral Reviews* 29: 1361–1373.

This article is also available in pdf format at http://www.annzool.net/

MOLECULAR ECOLOGY

Molecular Ecology (2013) 22, 5861–5876 doi: 10.1111/mec.12526

Quantitative trait loci for growth and body size in the nine-spined stickleback *Pungitius pungitius* L.

VERONIKA N. LAINE,* TAKAHITO SHIKANO,† GÁBOR HERCZEG,†‡ JOHANNA VILKKI§ and JUHA MERILÄ†

*Division of Genetics and Physiology, Department of Biology, University of Turku, Turku 20014, Finland, †Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, Helsinki, PO Box 65, 00014, Finland, ‡Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/C, 1117 Budapest, Hungary, §MTT Agrifood Research Finland, Jokioinen, Finland

Abstract

Body size is an ecologically important trait shown to be genetically variable both within and among different animal populations as revealed by quantitative genetic studies. However, few studies have looked into underlying genetic architecture of body size variability in the wild using genetic mapping methods. With the aid of quantitative trait loci (QTL) analyses based on 226 microsatellite markers, we mapped body size and growth rate traits in the nine-spined stickleback (*Pungitius pungitius*) using an F_2-intercross (n = 283 offspring) between size-divergent populations. In total, 17 QTL locations were detected. The proportion of phenotypic variation explained by individual body size-related QTL ranged from 3% to 12% and those related to growth parameters and increments from 3% to 10%. Several of the detected QTL affected either early or late growth. These results provide a solid starting point for more in depth investigations of structure and function of genomic regions involved in determination of body size in this popular model of ecological and evolutionary research.

Keywords: body size, growth, linkage map, QTL, quantitative trait loci

Received 8 July 2013; revision received 6 September 2013; accepted 11 September 2013

Introduction

Growth rate and body size are traits important for fitness as they influence survival and fecundity in a wide range of taxa (Arendt 1997). Quantitative genetic and common garden studies of various vertebrate species have revealed that both traits are moderately to highly heritable in most populations studied (e.g. Lynch & Walsh 1998; Gjedrem 2000), and genetically based divergence in mean growth rates and body size among locally adapted populations occurs often (e.g. Blanckenhorn 2000; Dmitriew 2011). Although these insights mostly derived from statistical quantitative genetic techniques have been very illuminating and useful in addressing many questions and problems of specialized- (e.g. James *et al.* 1997; Huey *et al.* 2000; Laurila *et al.* 2006) and even of more broad interest (e.g. Ellegren & Sheldon 2008), the mechanistic understanding of genetic architecture of quantitative traits requires identification of genes and genomic regions underlying expression of the traits of interest (Mackay *et al.* 2009; Hill & Kirkpatrick 2011).

Considering the ease with which different strains of fish can be crossed producing large number of progeny and thereby gaining large number of meiosis critical for genetic mapping (e.g. Darvasi & Soller 1997; Broman 2001), it is not surprising that in addition to studies in humans, model organisms (Flint & Mackay 2009), plants (e.g. Bergelson & Roux 2010) and domestic animal species (Andersson 2001; Andersson & Georges; Karlsson & Lindblad-Toh 2008), fish studies have spearheaded genetic mapping approaches [e.g. zebrafish, *Danio rerio* (Postlethwait *et al.* 1994 and Johnson *et al.* 1996) rainbow trout, *Oncorhynchus mykiss* (Young *et al.* 1998) medaka, *Oryzias latipes* (Wada *et al.* 1995) and tilapias (Kocher *et al.* 1998 and Agresti *et al.* 2000)]. This interest has been spurred by economic interest stemming from aquaculture, where the goal is often to produce fast-growing and large fish while minimizing the

Correspondence: Veronika N. Laine, Fax: +358 2 333 6598; E-mail: veronika.laine@utu.fi

costs (e.g. Canario *et al.* 2008). Likewise, the role of zebrafish as a model in developmental biology has paved the road for mapping studies in fish (Dahm & Geisler 2006; Lieschke & Currie 2007). However, it is only recently that evolutionary biologists have started to employ gene mapping approaches on vertebrate models to tackle questions relating to more conceptual problems (e.g. Peichel *et al.* 2001; Slate 2005; Wright *et al.* 2006; Slate *et al.* 2010; Hosoya *et al.* 2012).

The nine-spined stickleback (*Pungitius pungitius*) is an emerging model in ecological and evolutionary biology research (Merilä 2013). It has been the focus of numerous behavioural (e.g. Duffy *et al.* 2009; Herczeg *et al.* 2009a; Webster & Laland 2012), neuroanatomical (e.g. Trokovic *et al.* 2011; Gonda *et al.* 2012), developmental (e.g. Shapiro *et al.* 2006, 2009) and genetic (e.g. Aldenhoven *et al.* 2010; Shikano *et al.* 2010a,b,c; Teacher *et al.* 2011) studies. Recent studies have identified repeated evolution of 'giant-sized' nine-spined sticklebacks in northern Fennoscandian ponds (Herczeg *et al.* 2009b). Detailed quantitative genetic and common garden studies suggest additive genetic basis for this divergence (Herczeg *et al.* 2009b; Shimada *et al.* 2011; Ab Ghani *et al.* 2012). While body size is a trait likely to have polygenic basis, detailed comparisons of growth patterns in different populations of nine-spined sticklebacks (Shimada *et al.* 2011; Herczeg *et al.* 2012) suggest that size differences among populations could also have been achieved by relatively simple genetic mechanisms. Namely, when the growth of the fish from normal-sized marine population levels off, the pond fish continue their growth (Shimada *et al.* 2011; Herczeg *et al.* 2012). Hence, it is possible that a simple 'genetic switch' rather than complex polygenic inheritance underlies the population differences in body size.

The aim of the present study was to perform the first genome scan to locate possible QTL influencing growth and body size in the nine-spined stickleback. To this end, we used 283 F_2-generation full-sib offspring from mating between F_1-generation parents, which were offspring to a 'normal-sized' female parent from a marine population and to a 'giant-sized' male from pond population. A linkage map based on 226 microsatellite markers with an average intermarker interval of 8 cM was used for QTL detection (Shikano *et al.* 2013). Apart from mapping final body size, we also mapped sizes at different ages as well as growth curve parameters obtained from individually fitted von Bertalanffy functions.

Material and methods

Fish and phenotypic data

The fish forming the grand-parental generation (F_0) were collected in 2006 from a marine population from the Baltic Sea (Helsinki; 60°13′25″N, 25°11′09″E) and from a pond population in northeastern Finland (Rytilampi; 66°23′03″N, 29°19′12″E). The two populations have been subject to earlier studies, which have shown that the fish from these two populations are divergent in their body size and morphology, actually representing the extreme phenotypes found in these traits within nine-spined sticklebacks (Herczeg *et al.* 2009b, 2010).

A female fish from the marine population was crossed *in vitro* with a male from the pond population in July 2006, and the resulting F_1-offpring was group-reared in aquaria in 15 °C until they reached maturity. They were initially fed with *Artemia* nauplii and later on with frozen chironomid larvae *ad libitum*. After an artificial hibernation at 6 °C without light, fish were maintained at 17 °C under permanent light to facilitate reproduction. Once the F_1-fish had matured, one randomly selected male and female were chosen and allowed to mate repeatedly. In practice, the male was allowed to build a nest in a 20-L aquarium and the female was introduced to this aquaria whenever it appeared ripe (i.e. carrying eggs). After mating, the female was removed and kept separately until it was ripe again. This pair produced seven successive clutches. The clutches were removed from the male's nest and reared in 1.4-L tanks in Allentown zebrafish racks (Aquaneering Inc., San Diego, CA, USA; hereafter 'rack'). About 6 days after hatching, once the fry reached free-swimming stage and started feeding, they were individualized in to separate 1.4-L tanks of four racks. The fish were reared at 17 °C under a 14:10 light/dark photoperiod until they were 187 days old, after which they were anesthetized with an overdose of MS-222 (tricaine methanesulfonate). Due to juvenile mortality (mainly during the first month), we were able to collect data from 283 F_2 offspring at the end of the experiment.

Variation in growth was quantified with three different ways. First, we analysed size at different ages from standard length (SL, measured from the tip of the nose to the end of the tail base) data collected from digital photographs of each individual taken in following time points: 19, 47, 75, 103, 131 and 159 days (SL1, SL2, SL3, SL4, SL5, SL6) posthatch, respectively. These photographs were taken with a PANASONIC DMC-FZ8 digital camera from a tripod, using a standard set-up on every round. Standard length was measured from the digital photographs using tps.Dig 2.10 (Rohlf 2006) software. In the second method, three growth curve parameters were estimated as included in the following: initial fish length (L_0, mm), asymptotic fish length (L_{max}, mm) and growth constant (k, mm/day). These parameters were obtained by fitting von Bertalanffy growth curves to SL1-SL6 measures of every individual (von

Bertalanffy 1938). The growth constant is a relative measure of growth that indicates how fast size approaches the asymptote and it is linearly related to maximum growth rate on an absolute scale (mm/day, Aikio *et al.* 2013). We note that L_0 is not biologically informative in our case when growth was followed in a laboratory setting, so we did not analyse this variable. The growth model was fitted separately for each individual using nonlinear least squares method in software Statistica 6.1 (StatSoft Inc. Tulsa, OK, USA). The von Bertalanffy model gave a very good fit to individual growth data with coefficients of determination (R^2) ranging from 0.97 to 0.99. Finally, we analysed size changes between subsequent ages (growth increments: GR1, GR2, GR3, GR4, GR5; where GR1 = ((SL2-SL1)/SL1) × 100, GR2 = ((SL3-SL2)/SL2) × 100, etc.).

At the time of killing, the fresh weight of each fish was recorded and they were photographed laterally with a NIKON D60 digital camera. From these photographs, we calculated centroid size, which is the square root of the sum of the squared distances from the centroid of each landmark (Bookstein 1991) and is widely used as a reliable size proxy (e.g. Leinonen *et al.* 2006; Herczeg *et al.* 2010; Lee *et al.* 2011; Outomuro & Johansson 2011). The landmarks and procedures used for calculation of centroid size were described in Herczeg *et al.* (2010). All these calculations were made in software package tpsRelw 1.46 (Rohlf 2006) after placing the landmarks on the digital photographs with tps.Dig 2.10 (Rohlf 2006). Note that we did not include standard length measurements recorded at the time of killing for either the mapping analyses or for calculating the von Bertalanffy growth parameters for two reasons. First, all fish were subjected to behavioural assays between the last two sets of photographs (159 and 187 days post-hatching, respectively) including exploration tests outside of their home tanks and risk-taking tests including stressful stimuli in their home tanks (to be published separately). Such treatment can disrupt the growth of individuals, perhaps even in an individual-specific way . Second, the last set of photographs were taken with a different camera for the best possible image quality needed for the detailed shape analyses (to be published separately). The different cameras and their lenses had different distortion characteristics, which did not allow us to treat the pictures as directly comparable. Hence, we only used centroid size and body weight from the time of killing as proxies of 'final size'.

Genotyping

Total genomic DNA was extracted from ethanol-preserved pelvic fins using a silica-fines/microtitre filtration plate method (Elphinstone *et al.* 2003) following proteinase K digestion. All the F_2-offspring (n = 283), their parents (n = 2) and grand parents (n = 2) were genotyped for 235 loci, which were polymorphic in at least one of the parents. Based on the location of the markers in relation to annotated genes, ten markers were linked to growth-related genes (Shikano *et al.* 2010a), 36 markers were associated with other physiologically and behaviourally important genes (Shikano *et al.* 2010a; Shimada *et al.* 2011; Laine *et al.* 2012), whereas the rest of the markers were randomly selected (Largiadèr *et al.* 1999; Peichel *et al.* 2001; Heckel *et al.* 2002; Colosimo *et al.* 2004; Miller *et al.* 2007; Mäkinen *et al.* 2008; Shapiro *et al.* 2009; Shikano *et al.* 2011; Appendix I). The genotyped loci, together with information on PCR conditions are detailed in Shikano *et al.* (2013).

Linkage analyses and QTL mapping

The marker arrangement in the linkage map was determined using CRI-MAP software (version 2.5) (Green *et al.* 1990). The option TWOPOINT was used to obtain the logarithm of the odds (LOD) score for every pair of markers. The LOD score threshold of three (3) was used as a criterion of significance for linkage. In addition, earlier nine-spined stickleback linkage map (Shapiro *et al.* 2009) and three-spined stickleback (*Gasterosteus aculeatus*) genome information from Ensembl (genebuild May 2010, database version 66.1) were used as references. Option BUILD was used to determine the best order of the markers for each linkage group by beginning with the most informative marker pair. Markers that could not be fitted straight with BUILD and with LOD score ≥4.0 were fitted manually. The FLIPS option (N = 3–5) was used to evaluate the statistical significance of the obtained order. After the best order was determined within each linkage group, double recombination events were detected using the CHROMPIC option. Individuals with over four recombinations were removed and a second CRIMAP analysis round was conducted. Of the 235 markers genotyped, nine with low polymorphism levels were discarded due to low LOD scores in TWOPOINT. The final order for each linkage group is presented in Appendix I.

A total of 226 markers were used in QTL analyses and interval mapping was performed in GridQTL (Seaton *et al.* 2006; available at http://www.gridqtl.org.uk/) using the BC-F2 portlet that is based on the least squares regression method (Haley *et al.* 1994). Both additive and dominance effects were fitted, and analyses were performed at 1 cM intervals. As there were sex differences in centroid size (t_{281} = 3.51, P = 0.005), SL5 (t_{277} = 2.92, P = 0.004), SL6 (t_{277} = 3.16, P = 0.002), L_{max} (t_{274} = 2.06, P = 0.040) and GR4 (t_{276} = 276, P = 0.032), sex was used as a fixed effect when mapping these traits. In addition, clutch differences were detected in every variable (P < 0.03) except SL2 and L_{max}. Hence, clutch identity was also fitted as a fixed effect.

Chromosome- and experiment-wide significance levels were determined by using permutation tests with 10 000 iterations (Churchill & Doerge 1994; Doerge & Churchill 1996). A QTL was considered significant when the F-value was above the 5% experiment-wide threshold and suggestive when it was above the 5% chromosome-wide threshold (Lander & Kruglyak 1995). Confidence intervals were obtained with bootstrap analysis with 10 000 iterations. The percentage of the phenotypic variance explained (PVE) by the QTL was calculated following Zhou *et al.* (2006).

Results

Phenotypic variation

The mean values and associated dispersion measures for all mapped phenotypic traits are given in Table 1. The proxies of final body size (*viz.* body weight and centroid size) were correlated (Table 2), but the variability in body weight, as reflected in it is high coefficient of variation, was higher than that of centroid size (Table 1). Standard lengths at different ages were also strongly correlated, especially among adjacent time points with a tendency towards decreasing correlations with time (Table 2). Growth curve parameters and growth increments tended to show higher coefficient of variations than the linear size measurements (Table 1).

Body size QTL

QTL for final body size (body weight, centroid size) and standard length measured at different time points are presented in Tables 3a and 4. Altogether 19 genomic locations dispersed over 13 linkage groups (LGs) showed association with body size, and from these, nine QTL in three LGs (8, 12 and 13) were significant (Tables 3a and 4). At two locations, the significant QTL for different measurements of body size located at exactly the same position: traits SL1 and SL2 on LG 12 (4 cM) and traits centroid size, SL4, SL5 and SL6 in LG 13 (82 cM). The variance explained by the individual QTL varied from 3% to 12% (Tables 3a and 4). The highest LOD score was found in LG 12 for trait SL1. Additive effects were detected more frequently than dominance effects (Table 3a). QTL with dominance effects were found in the LGs 1 (SL4), 5 (SL6) and 16 (SL1, SL2, SL3; Table 3a). Looking at the total variance in different traits explained by detected QTL, up to 34% of the phenotypic variance in SL2 was explained, whereas for centroid size, this value was 8%. A comparison of effects between genotypes of two significant QTL peaks, Ppgm2/LG 8 and Pun173/LG 13 is shown

Table 1 Means and associated dispersion estimates for mapped body size and growth traits in nine-spined sticklebacks

Trait	Mean	SD	n	Min	Max	CV (%)
Size traits at the end of the experiment						
Body weight (BW; g)	1.07	0.20	283	0.53	1.76	19
Centroid size (Csize)	6.27	0.54	283	4.82	7.77	9
Size at different ages (mm)						
SL1 (19 d)	11.94	0.74	282	9.87	14.12	6
SL2 (47 d)	23.72	2.04	281	19.68	29.82	9
SL3 (75 d)	32.24	2.61	280	26.42	40.28	8
SL4 (103 d)	41.56	3.08	280	31.97	51.85	7
SL5 (131 d)	46.52	3.29	279	34.64	55.75	7
SL6 (159 d)	48.96	3.83	279	38.63	59.08	8
Growth curve parameters						
Asymptotic size, L_{max} (mm)	64.54	10.86	276	40.71	103.65	17
Growth constant, k (mm/d)	0.07	0.018	276	0.025	0.131	26
Growth increments						
GR1 (19–47 d)	98.86	13.74	281	71.46	159.87	14
GR2 (47–75 d)	36.19	8.73	280	10.69	59.62	24
GR3 (75–103 d)	28.97	5.85	279	6.29	45.62	20
GR4 (103–131 d)	12.21	4.64	278	1.96	44.39	38
GR5 (131–159 d)	5.15	2.48	277	−0.69	13.42	48

SD, standard deviation; n, number of individuals; Min and Max, minimum and maximum value, respectively; CV(%) = coefficient of variation.

Table 2 Phenotypic correlation coefficients (Pearson product-moment) among growth and body size traits used for quantitative trait loci (QTL) mapping

	BW	Csize	SL1	SL2	SL3	SL4	SL5	SL6	L_{max}	k	GR1	GR2	GR3	GR4	GR5
BW	1														
Csize	**0.79**	1													
SL1	**0.29**	**0.26**	1												
SL2	**0.25**	**0.44**	**0.63**	1											
SL3	**0.44**	**0.64**	**0.41**	**0.71**	1										
SL4	**0.51**	**0.77**	**0.26**	**0.56**	**0.85**	1									
SL5	**0.69**	**0.92**	**0.32**	**0.53**	**0.73**	**0.87**	1								
SL6	**0.77**	**0.96**	**0.32**	**0.50**	**0.68**	**0.80**	**0.95**	1							
L_{max}	**0.65**	**0.68**	0.06	−0.06	−0.01	**0.21**	**0.56**	**0.67**	1						
k	**−0.40**	**−0.29**	0.04	**0.40**	**0.46**	**0.24**	−0.16	**−0.28**	**−0.82**	1					
GR1	0.04	**0.31**	−0.12	**0.70**	**0.52**	**0.47**	**0.38**	**0.33**	−0.13	**0.48**	1				
GR2	**0.21**	**0.20**	**−0.35**	**−0.45**	**0.31**	**0.31**	**0.20**	**0.18**	0.06	0.04	**−0.26**	1			
GR3	0.02	0.09	**−0.31**	**−0.36**	**−0.40**	0.12	0.12	0.06	**0.34**	**−0.40**	−0.16	−0.02	1		
GR4	**0.30**	**0.24**	0.08	−0.08	**−0.27**	**−0.29**	**0.18**	**0.24**	**0.64**	**−0.73**	**−0.18**	**−0.24**	−0.10	1	
GR5	**0.48**	**0.41**	0.11	0.07	0.08	0.03	0.14	**0.42**	**0.53**	**−0.45**	−0.02	−0.01	−0.15	**0.24**	1

Values in bold are significant after sequential Bonferroni correction ($P < 0.005$). For trait abbreviations, see Table 1.

in Table 5. In both cases, alleles coming from the 'giant' pond population increased size and body weight of individuals (Table 5).

Growth curve parameters and growth increments QTL

Fewer QTL were detected for growth curve parameters (L_{max} and k) and growth increments (GRs) than for body size traits, and most of them were merely suggestive (Tables 3b and 4). Significant QTL were observed in LGs 7 (GR7) and 8 (GR1 and GR2; Table 3b). Variance explained by individual QTL varied from 3% to 10 % and total variance in different traits from 3% to 32 %. Dominance effects were observed in LGs 5, 9 and 12 (Table 3b).

Comparison of body size, growth curve parameters and growth increment QTL

All QTL locations for body size, growth curve parameters and growth increments are presented in Table 4. Later, stage body size (SL4-6) QTL locations were similar with most of the final body size QTL (centroid size). However, fewer QTL were detected for later stage body size (SL4-6) than for the early stage body size (SL1-3; Table 4). QTL locations for early and later stage body size were different. In early size stages, especially LGs 4, 8, 12 and 16 were contributing, while LGs 7 and 13 were important in the later stages. When comparing body size from different time points (SL1-6) and growth increment (GR1-5) QTL with each other, some of the QTL were overlapping. Genetic effect was found in LG 8 both at early size stages and GR1 and GR2. In contrast, a strong genetic impact was found in LG 7 at GR3 and this effect also remained in later stage body sizes (SL4-6).

Candidate gene markers for body size and growth

In many cases, the 95% confidence interval covered a large area of the linkage group encompassing the positions where growth, behavioural and physiological gene markers were indicated to be located (Table 3). From the specific QTL locations, one marker (Ppgm2) was related with growth, two with behaviour (Ppbig2 and Ppbig5) and four with the physiology (ATP1A1, NHE2b, Ppgm12 and Ppgm40; Table 3; markers related with behaviour and physiology are shown in Appendix I).

Discussion

Body size is an ecologically important trait and its genetic basis is thought to be highly polygenic in most organisms. Earlier studies of nine-spined sticklebacks have confirmed heritable basis in this trait (Shimada *et al.* 2011) and also showed it to be genetically based among populations (Herczeg *et al.* 2009b; Herczeg *et al.* 2012; Ab Ghani *et al.* 2012). Here, we have identified several significant and suggestive QTL, which influence variation in body size and related growth traits. As opposed to the hypothesis of a simple major gene-based 'switch'-like genetic architecture of body size control in this species (see Introduction), the results support the contention that many genes with small effects underlie within body size variability. The results further suggest that different genes are likely to influence variation in body size at different ages with both additive and non-

Table 3 Estimates of linkage group (LG) location, 95% confidence intervals (CI), significance (F & LOD), chromosome and experiment-wide thresholds for both 1% and 5% levels, additive and dominance effects (±SE; dominance effects in bold are significant with $P < 0.05$), percentage of variation explained by individual QTL (PVE) and all QTL for given trait for body size (a) and growth increment (b)-related QTL in *Pungitius pungitius*

Trait	LG	Location (cM)		F	LOD	CI	Chromosome wide (0.01)	Chromosome wide (0.05)	Experiment wide (0.01)	Experiment wide (0.05)	Additive effect (SE)	Dominance effect (SE)	PVE	Total PVE/trait	Closest marker
(a)															
BW	1	2		6.92	2.93	0.0–118.0	7.57	5.64	10.51	8.48	−0.05 (0.02)	0.06 (0.02)	4	16	Pun3/Pun206
	13	14		**13.57**	5.61	5.0–92.0	7.16	5.35			**−0.10 (0.02)**	**−0.02 (0.03)**	9		Pun163
	16	55		5.32	2.27	12.0–95.0	7.06	5.17			−0.06 (0.02)	−0.05 (0.03)	3		Pprm14
Csize	**13**	82		**13.03**	5.40	2.0–92.0	7.34	5.44	10.54	8.50	−0.24 (0.05)	−0.09 (0.06)	8	8	Pun173
SL1	4	121		5.98	2.54	10.0–121.0	7.14	5.41	10.27	8.45	−0.23 (0.10)	−0.06 (0.11)	3	33	Pun272
	8	0		**10.25**	4.29	0.0–51.0	6.64	4.88			−0.24 (0.06)	0.06 (0.08)	6		Stn89
	9	78		8.37	3.53	54.0–79.0	6.84	5.04			0.24 (0.06)	0.07 (0.09)	5		Pun257
	12	4		**19.89**	8.07	1.0–17.0	7.42	5.48			−0.33 (0.06)	0.28 (0.08)	12		*Ppgm12*
	16	55		6.29	2.67	5.0–80.5	7.17	5.28			−0.15 (0.07)	**−0.30 (0.11)**	4		Pprm14
	17	0		5.98	2.54	0.0–36.0	6.31	4.43			−0.21 (0.06)	0.01 (0.08)	3		Umf10
SL2	4	121		6.25	2.65	7.0–121.0	7.45	5.48	10.33	8.50	−0.91 (0.29)	0.25 (0.33)	4	34	Pun272
	5	46		8.38	3.54	9.0–57.0	6.60	4.91			−0.67 (0.19)	−0.73 (0.27)	5		Pun286
	8	22		**14.52**	6.00	0.0–50.0	6.46	4.72			−0.91 (0.17)	0.11 (0.24)	9		*Ppgm2*
	11	59		6.53	2.77	0.0–75.0	7.16	5.36			−0.56 (0.18)	0.60 (0.27)	4		Pun185
	12	4		**9.00**	3.79	0.0–95.0	7.23	5.41			−0.70 (0.17)	0.45 (0.25)	5		*Ppgm12*
	15	30		5.24	2.24	9.0–67.0	7.13	4.85			0.67 (0.21)	0.12 (0.33)	3		Umf38
	16	39		6.14	2.61	5.0–59.0	6.99	5.21			−0.20 (0.18)	**−0.88 (0.28)**	4		Umf44
SL3	5	47		7.66	3.24	7.0–57.0	6.73	4.88	10.25	8.36	−0.76 (0.23)	−0.91 (0.34)	5	19	Pun286
	8	8		6.04	2.57	0.0–51.0	6.56	4.70			−0.64 (0.20)	−0.48 (0.30)	4		Pun68
	10	44		5.35	2.28	0.0–49.0	6.60	4.71			−0.67 (0.21)	−0.29 (0.29)	3		Pun42
	13	82		6.50	2.76	0.0–98.0	7.25	5.43			−0.80 (0.22)	−0.34 (0.30)	4		Pun173
	16	27		5.42	2.31	4.0–81.0	7.03	5.20			−0.36 (0.22)	**−0.90 (0.35)**	3		*Ppbig2*
SL4	1	3		6.23	2.64	0.0–137.0	7.39	5.53	10.09	8.48	0.07 (0.24)	**1.20 (0.34)**	4	17	Pun206
	7	15		8.06	3.40	7.0–57.0	4.72	6.49			−0.90 (0.23)	0.30 (0.36)	5		*Ppbig5*
	13	82		**12.90**	5.35	8.0–98.0	7.25	5.42			−1.26 (0.25)	−0.28 (0.33)	8		Pun173
SL5	7	16		7.20	3.05	2.0–57.0	6.62	4.74	10.24	8.46	−1.01 (0.27)	−0.009 (0.43)	4	12	*Ppbig5*
	13	82		**13.18**	5.46	5.0–92.0	7.40	5.53			−1.48 (0.29)	−0.48 (0.39)	8		Pun173
SL6	5	50		5.82	2.47	0.0–80.0	6.72	4.96	10.19	8.44	**−0.98 (0.38)**	**−1.50 (0.59)**	3	16	Pun286
	7	16		4.69	2.00	3.0–56.0	6.39	4.66			−0.96 (0.31)	−0.15 (0.51)	3		*Ppbig5*
	13	82		**16.17**	6.63	6.0–92.0	7.31	5.52			−1.85 (0.33)	−0.78 (0.45)	10		Pun173

Table 4 Distribution of QTL related to growth and body size across the 21 linkage groups (LG) indicating the chromosome- (light grey blocks) and experiment-(dark grey blocks) wide significance levels

Trait abbreviations as in Table 1.

Table 5 Comparison of (a) standard length traits (SL, in mm) and (b) body size traits among genotypes of markers Ppgm2/LG 8 (early stage size) and Pun173/LG 13 (later stage size)

(a) Ppgm2/LG8	SL1	SL2	SL3
MM	11.70 (0.68)	22.82 (2.14)	31.63 (2.38)
MP	11.98 (0.77)	23.83 (1.87)	32.30 (2.42)
PP	**12.11 (0.68)**	**24.64 (1.93)**	**32.90 (3.22)**

(b) Pun173/LG13	BW (g)	Csize	SL4	SL5	SL6
Mm	1.01 (0.20)	6.13 (2.95)	40.53 (3.24)	45.54 (3.60)	47.86 (0.43)
MP	1.05 (0.18)	6.21 (3.16)	41.48 (3.55)	46.19 (4.11)	48.49 (0.44)
mP	1.09 (0.19)	6.27 (2.87)	41.69 (2.73)	46.59 (3.21)	48.95 (0.38)
PP	**1.16 (0.24)**	**6.55 (3.17)**	**42.95 (3.36)**	**48.38 (3.91)**	**51.29 (0.40)**

Mm alleles from marine, Pp alleles from pond. Largest values in bold.

additive influences. In the following, we will discuss the implications of these findings to our understanding of the genetic basis of body size variation in fishes and relate them to what is known about genetics of growth-related traits in other species.

Genetic determination of growth

Variation in many of the body size and growth-related traits mapped to similar QTL locations suggesting that same genetic factors may govern variability in these traits. This is not surprising in the view that some of the mapped variables can be viewed as different proxies of same traits (e.g. standard length and centroid size). However, more noteworthy is the fact that the QTL locations for early and later stage body sizes often resided in different linkage groups; QTL for traits SL1-3 often resided in the same linkage groups, whereas QTL for SL4-6 (as well as for centroid size) were identified mainly in other areas. These results suggest that distinct sets of genetic factors influencing size are active at different ages. This phenomenon has been also observed

Table 3 *Continued*

Trait	LG	Location (cM)	F	LOD	CI	Chromosome wide (0.01)	Chromosome wide (0.05)	Experiment wide (0.01)	Experiment wide (0.05)	Additive effect (SE)	Dominance effect (SE)	PVE	Total PVE/trait	Closest marker
(b)														
L_{max}	2	27	7.80	3.29	6.0–71.0	6.72	4.96	10.32	8.42	−4.15 (1.06)	−0.88 (1.85)	5	13	*NHE2b*
	10	27	4.79	2.05	0.0–55.0	6.69	4.73			2.23 (0.96)	2.83 (1.31)	3		Pun156
k	1	98	6.20	2.63	0.0–105.0	7.33	5.55	10.22	8.36	0.005 (0.002)	−0.0002 (0.002)	4	13	Umf48
	13	82	7.85	3.31	3.0–98.0	7.30	5.34			−3.55 (0.99)	−2.74 (1.33)	5		Pun173
	2	42	5.82	2.48	12.0–63.0	6.85	4.95			0.005 (0.002)	0.002 (0.002)	3		Pun96
	8	22	5.02	2.14	0.0–51.0	6.69	4.83			−0.005 (0.002)	−0.001 (0.002)	3		*Ppgm2*
	17	34	4.92	2.10	0.0–36.0	6.14	4.41			−0.005 (0.002)	−0.001 (0.002)	3		Pun233
GR1	5	51	7.89	3.33	9.0–58.0	6.80	4.91	10.22	8.38	−3.70 (1.33)	**−6.71 (2.10)**	5	21	Pun286
	8	**23**	**9.16**	3.85	0.0–49.0	6.60	4.71			−4.80 (1.14)	0.55 (1.61)	6		*Ppgm2*
	9	51	6.17	2.62	0.0–79.0	6.92	5.12			−1.16 (1.24)	**6.08 (1.82)**	4		Ppgm61
	12	85	5.50	2.34	25.0–117.0	7.06	5.34			**−0.73 (1.10)**	**−5.03 (1.58)**	3		Tpsm6
GR2	1	82	7.53	3.18	0.0–122.0	7.50	5.60	10.42	8.46	2.60 (0.70)	1.70 (1.19)	5	32	Pun49
	18	78	5.33	2.27	9.0–79.0	6.56	4.91			2.73 (1.12)	−3.86 (1.70)	3		Stn195
	8	**22**	**15.47**	6.36	18.0–38.0	6.55	4.69			3.32 (0.62)	−0.68 (0.85)	10		*Ppgm2*
	11	41	7.98	3.37	1.0–62.0	7.21	5.36			2.43 (0.61)	−0.66 (0.91)	5		*Ppgm40*
	12	95	6.87	2.91	0.0–107.0	7.32	5.50			2.23 (0.61)	0.44 (0.88)	4		Pun2
GR3	14	17	7.89	3.33	6.0–69.0	6.59	4.84			2.24 (0.60)	−1.31 (0.87)	5		Pun203
	7	56	**12.35**	5.13	51.0–57.0	6.45	4.70	10.24	8.44	−2.09 (0.45)	1.28 (0.66)	8	16	Pun319
	10	42	8.29	3.49	25.0–49.5	6.71	4.74			2.00 (0.49)	0.04 (0.70)	5		Pun42
	17	25	4.98	2.12	0.0–36.0	6.26	4.39			1.43 (0.51)	1.25 (0.85)	3		Pun233
GR4	2	27	5.28	2.25	11.0–55.0	6.47	4.77	10.03	8.14	**−1.21 (0.39)**	**−0.62 (0.68)**	3	3	*NHE2b*
GR5	1	89	7.71	3.26	3.0–137.0	7.40	5.58	10.39	8.38	−0.80 (0.21)	−0.31 (0.31)	5	8	*ATPIA1*
	15	68	5.29	2.26	7.0–68.0	6.63	4.84			0.68 (0.21)	0.04 (0.29)	3		Pun159

LGs and F-values in bold are significant at experiment-wide level and the remaining values are suggestive QTL (chromosome-wide significance).
Gene-related marker in italics. All estimates refer to sex-averaged results.

in studies of many model and domestic animals, such as in mice (e.g. Cheverud *et al.* 1996; Rocha *et al.* 2004; Allan *et al.* 2005) and chickens (Carlborg *et al.* 2003; Podisi *et al.* 2013). Cheverud *et al.* (1996) observed dominance (and/or overdominance) for early growth, and they suggested that it may have developed as a response to selection for an increased early growth rate, which can be an important component of fitness in mice. This dominance at early stages was confirmed by Rocha *et al.* (2004), who observed increased importance of additive effects towards maturity. At the same time, importance of dominance effects decreased, with reversal of the direction of dominance in many loci (Rocha *et al.* 2004). In our case, only a few QTL with dominance effects were detected, and they were mostly found in the early stage body size traits. Additive gene effects were more frequent in our results, and this is consistent with the findings of an earlier quantitative genetic study of body size divergence in Fennoscandian nine-spined sticklebacks (Ab Ghani *et al.* 2012). Similarly from conclusions of Ab Ghani *et al.* (2012) study, alleles from the paternal grandparent (pond population) increased body size both at the early and late stages of development. Hence, the QTL mapping results align with results of earlier quantitative genetic studies suggesting that growth and body size differences among pond and marine populations owe to genetic changes that have occurred in the pond populations.

Typical for many mapping studies of growth-related traits is that many QTL with small effects are detected (e.g. Andersson & Georges 2004; Rocha *et al.* 2004), and this was found true in the present study also. Fewer QTL were observed in the later as compared with early growth stages suggesting that environmental effects may have a larger impact on growth at later stages (cf. Podisi *et al.* 2013). However, because the fish used in this study were 2nd generation laboratory fish reared in standardized environmental conditions, the influence of direct and systematic environmental effects are unlikely. It has been demonstrated in rodents that different physiological mechanisms are affecting growth at different life stages. Early growth in most tissues is caused by dividing cells, whereas growth in later stages is primarily due to an increase in cell size, especially in neural, muscular and adipose tissues (Atchley *et al.* 1984; Riska *et al.* 1984; Atchley & Zhu 1997). Hence, environmental 'noise' due to random variation in growth and allocation patterns might increase towards later growth stages and explain the decreasing number of QTL with age. This conjecture is supported by the low phenotypic correlations between early and later stage sizes. Hence, the growth and development of body size in nine-spined sticklebacks at different stages of development may be influenced by different genetic mechanisms (QTL), but also by changes in relative importance of environmental and genetic sources of variation during the ontogeny.

Mapping growth curve parameters and growth increments uncovered less QTL than the mapping of actual size traits. This may suggest there are only a few major areas in the genome that are control growth patterns, a finding which has been reported to also apply to chicken (Podisi *et al.* 2013). However, we note that smaller effect QTL might have gone undetected due to lack of statistical power to detect weak effects. Some of the growth increment QTL overlapped with the QTL for actual size traits. For instance, in LG 8, a significant genetic effect was found at early growth increments in addition to two significant QTL in SL1 and SL2. In addition, a strong QTL was detected in LG 7 for GR3 accompanied by suggestive QTL for SL4-6 traits in the same linkage group but in different location. A significant QTL for the last growth increment (GR5) mapped into LG 1, where also QTL for growth constant, *k*, and final body weight resided, albeit apparently in a different location. These results suggest that by and large, different parts of the genome are influencing phenotypic variation in the growth patterns and the measurements of size at a given time.

Comparison with behaviour QTL

When comparing these results with the earlier QTL analyses performed with behavioural traits on the same populations (V. N. Laine, G. Herczeg, T. Shikano, J. Vilkki, J. Merilä, submitted), it was noticed that many QTL areas for body size and behavioural traits were overlapping. While this might be because of the wide confidence intervals for detected QTL regions, it is also possible that this could indicate tight linkage of QTL or pleiotropic effects on body size and behavioural variation. A particularly interesting case is provided by the significant QTL in LG8 close to gene-related marker Ppgm2 detected in both studies. This marker is located close to pituitary adenylate cyclase-activating polypeptide (*ADCYAP1 / ACAPRb*) gene known to have functions during growth (Lugo *et al.* 2008) as well as in post-traumatic stress disorders (Ressler *et al.* 2011), and hence, it provides a candidate gene for explaining variation in exploration-related behaviour and early life body size. A connection between size and behaviour QTL has been also noticed in earlier studies conducted on fowl (Schütz *et al.* 2004; Wirén *et al.* 2013). Likewise, in trout (*Salmo trutta*), variation in boldness and exploration has been linked to variation in growth rates (Lahti *et al.* 2002; Adriaenssens & Johnsson 2010; Biro & Stamps 2010). The biological link can also be as straightforward in nine-spined sticklebacks. As fast growth and larger size both rely on high rates of energy consumption, which in turn rely on high behavioural activity, studying the proximate links between size/growth and behaviour could be rewarding as genetic variation in growth rates and patterns may be mediated by genetic factors influencing behaviour.

Body size in other fish species

Body size QTL have been analysed in many fish species (reviewed in Hosoya *et al.* 2012). As compared with earlier work conducted on sticklebacks, our results are comparable with those of Albert *et al.* (2008) who identified a single significant size-related QTL in three-spined stickleback, using a linkage map containing 248 microsatellite markers applied to 372 individuals from an F_2-cross. In that study, standard length mapped to one location in LG 13. Also in our analysis, significant QTL for centroid size and body weight were mapped to LG 13 with 8% and 9 % of phenotypic variance explained, respectively. In contrast, an earlier three-spined stickleback study (Colosimo *et al.* 2004) using 160 microsatellite markers and 360 individuals mapped body size to LG 19 with an effect size of 13%. Both of these three-spined-stickleback studies used similar population crosses and similar numbers of individuals for QTL mapping. One possible explanation for these differing results is that the mapping cross used in the present study was created from populations that were more divergent in body size than those used by Colosimo *et al.* (2004) and Albert *et al.* (2008) (discussed more below).

When comparing the numbers of growth and body size-related QTL in fish, two kinds of outcomes are identifiable: fish with single major QTL for size or many QTL with small or intermediate effects. However, there are also cases where major QTL fractionate into smaller QTL when a finer scale mapping is performed (e.g. Noor *et al.* 2001; Studer & Doebley 2011). A recent study by Hosoya *et al.* (2012) compared growth rates in two species of pufferfish (*Takifugu* spp.), and juvenile body size was mapped to one QTL location suggesting one major gene mode of inheritance. In contrast, several QTL were found to influence body size variation in the Mexican cavefish (*Astyanax mexicanus*; Protas *et al.* 2008) and the lake whitefish (*Coregonus clupeaformis*; Rogers & Bernatchez 2007). Hence, the above-mentioned three-spined stickleback studies together with Hosoya *et al.*'s (2012) pufferfishes and the nine-spined sticklebacks used here are putative examples of single QTL influences on body size variation. In contrast, studies of cavefish and whitefish are examples of many loci affecting body size. There are two possible explanations for these contrasting results. First, there may be real differences in genetic architecture of body size in different species: depending on the details of species evolutionary history, the distribution effect sizes of loci underlying quantitative trait variation may be expected to differ (Yeaman & Whitlock 2011). However, more fine-scaled mapping studies are needed to draw any definitive conclusions with respect to this possibility. The same applies to similarity between QTL effects with respect to size in three- and nine-spined sticklebacks: although a major QTL influencing body size in both species is located in the same linkage group (LG 13; Albert *et al.* 2008; this study), it would be too early to conclude that the same genetic factors influence body size variation in these species, which diverged about 10 million years ago (Bell & Foster 1994).

The second possibility – which is also perhaps more plausible – is that the apparent differences in genetic architecture are driven by experimental design effects specific to each study. When sample sizes are modest, the QTL effects are often biased upward and small QTL are not detected (Beavis effect: Beavis 1994, 1998; see also: Xu 2003). Increasing the family sizes in the pufferfish (Hosoya *et al.* 2012) and three-spined stickleback (Colosimo *et al.* 2004; Albert *et al.* 2008) cases would help to see if there are yet undetected small effect QTL affecting body size, and also, why there are differences in the identity of LGs influencing body size variation between the two three-spined stickleback studies. However, in the case of our study, the power to detect a QTL explaining 10% of phenotypic variation is almost 100%, and the power to detect a QTL explaining 2% is over 40% (calculations based on procedures described in Rebai *et al.* 1995). Therefore, we should have had reasonable statistical power to detect QTL with even quite small effects. Furthermore, it should be noted that the studies by Albert *et al.* (2008) and Colosimo *et al.* (2004) were geared towards detecting body shape and armour QTL, respectively, and hence, the choice of their study populations was perhaps not optimal for mapping body size QTL. Hence, QTL studies specifically focused on body size QTL (i.e. using parental populations with large divergence in adult size) in three-spined sticklebacks are still to be conducted to make fair comparison to our results.

Conclusions

To sum up, we have identified a number of QTL – spread across the nine-spined stickleback linkage map – affecting variation in growth and body size in this species showing large interpopulation differentiation in mean body size. Furthermore, colocalization of the QTL in similar chromosomal regions suggests a role for pleiotropy and/or tight linkage explaining genetic basis of body size variation. The results also indicate that partially different genomic regions are responsible for controlling early and late body size stages in nine-

spined stickleback. Furthermore, loci affecting size and growth traits were located in some cases in the same chromosomal regions as loci affecting behaviour in the nine-spined stickleback. In general, the results lay the foundations for the fine mapping of these traits and provide a starting point for the identification of genes responsible for the size differences between marine and pond nine-spined sticklebacks.

Acknowledgements

We thank Abigel Gonda, Mirva Turtianen and Pirkko Siikamäki (the Oulanka Research Station; University of Oulu) for help in obtaining the fish; Kirsi Kähkönen for help in genotyping; and Thomas Lilley for comments that improved an earlier version of this manuscript. Our research was supported by Academy of Finland (Grant numbers 34728, 250435, 218343 to J.M. and 128716 to G.H.) and the Biological Interactions Graduate School (V.N.L.). GH also received financial support from the Hungarian Scientific Research Fund (OTKA-K 105517) and was supported by the János Bólyai Research Scholarship of the Hungarian Academy of Sciences. The experiments carried out in this study were conducted under license from the Finnish National Animal Experiment Board (Id # STH397A and STH223A), and the fish from Kuusamo were obtained with license from Metsähallitus.

References

Ab Ghani NI, Herczeg G, Merilä J (2012) Body size divergence in nine-spined sticklebacks: disentangling additive genetic and maternal effects. *Biological Journal of the Linnean Society*, **107**, 521–528.

Adriaenssens B, Johnsson JI (2010) Shy trout grow faster: exploring links between personality and fitness-related traits in the wild. *Behavioral Ecology*, **22**, 135–143.

Agresti JJ, Seki S, Cnaani A *et al.* (2000) Breeding new strains of tilapia: development of an artificial center of origin and linkage map based on AFLP and microsatellite loci. *Aquaculture*, **185**, 43–56.

Aikio S, Herczeg G, Kuparinen A, Merilä J (2013) Optimal growth strategies under divergent predation pressure. *Journal of Fish Biology*, **82**, 318–331.

Albert AYK, Sawaya S, Vines TH *et al.* (2008) The genetics of adaptive shape shift in stickleback: pleiotropy and effect size. *Evolution*, **62**, 76–85.

Aldenhoven JT, Miller MA, Corneli PS, Shapiro MD (2010) Phylogeography of ninespine sticklebacks (*Pungitius pungitius*) in North America: glacial refugia and the origins of adaptive traits. *Molecular Ecology*, **19**, 4061–4076.

Allan MF, Eisen EJ, Pomp D (2005) Genomic mapping of direct and correlated responses to long-term selection for rapid growth rate in mice. *Genetics*, **170**, 1863–1877.

Andersson L (2001) Genetic dissection of phenotypic diversity in farm animals. *Nature Reviews Genetics*, **2**, 130–138.

Andersson L, Georges M (2004) Domestic-animal genomics: deciphering the genetics of complex traits. *Nature Reviews Genetics*, **5**, 202–212.

Arendt JD (1997) Adaptive intrinsic growth rates: an integration across taxa. *Quarterly Review of Biology*, **72**, 149–177.

Atchley WR, Zhu J (1997) Developmental quantitative genetics, conditional epigenetic variability and growth in mice. *Genetics*, **147**, 765–776.

Atchley WR, Riska B, Kohn LAP, Plummer AA, Rutledge JJ (1984) A quantitative genetic analysis of brain and body size associations, their origin and ontogeny: data from mice. *Evolution*, **38**, 1165–1179.

Beavis WD (1994) The power and deceit of QTL experiments: lessons from comparative QTL studies. In: *Proceedings of the Forty-Ninth Annual Corn & Sorghum Industry Research Conference*, pp. 250–266. American Seed Trade Association, Washington, DC.

Beavis WD (1998) QTL analyses: power, precision, and accuracy. In: *Molecular Dissection of Complex Traits* (ed. Paterson AH), pp. 145–162. CRC Press, New York.

Bell AM, Foster S (1994) *The Evolutionary Biology of the Threespine Stickleback*. Oxford University Press, Oxford.

Bergelson J, Roux F (2010) Towards identifying genes underlying ecologically relevant traits in *Arabidopsis thaliana*. *Nature Reviews Genetics*, **11**, 867–879.

von Bertalanffy L (1938) A quantitative theory of organic growth (inquires on growth laws II). *Human Biology*, **10**, 181–213.

Biro PA, Stamps JA (2010) Do consistent individual differences in metabolic rate promote consistent individual differences in behavior? *Trends in Ecology and Evolution*, **25**, 653–659.

Blanckenhorn WU (2000) The evolution of body size: what keeps organisms small? *The Quarterly Review of Biology*, **75**, 385–407.

Bookstein FL (1991) *Morphometric Tools for Landmark Data: Geometry and Biology*. Cambridge University Press, Cambridge.

Broman KW (2001) Review of statistical methods for QTL mapping in experimental crosses. *Lab Animal*, **30**, 44–52.

Canario AVM, Bargelloni L, Volckaert F, Houston RD, Massault C, Guiguen Y (2008) Genomics toolbox for farmed fish. *Reviews in Fisheries Science*, **16**, 3–15.

Carlborg O, Kerje S, Schütz K, Jacobsson L, Jensen P, Andersson L (2003) A global search reveals epistatic interaction between QTL for early growth in the chicken. *Genome Research*, **13**, 413–421.

Cheverud JM, Routman EJ, Duarte FAM, van Swinderen B, Cothran K, Perel C (1996) Quantitative trait loci for murine growth. *Genetics*, **142**, 1305–1319.

Churchill GA, Doerge RW (1994) Empirical threshold values for quantitative trait mapping. *Genetics*, **138**, 963–971.

Colosimo PF, Peichel CL, Nereng K *et al.* (2004) The genetic architecture of parallel armor plate reduction in threespine sticklebacks. *PLoS Biology*, **2**, 635–641.

Dahm R, Geisler R (2006) Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. *Marine Biotechnology*, **8**, 329–345.

Darvasi A, Soller M (1997) A simple method to calculate resolving power and confidence interval of QTL map location. *Behavior Genetics*, **27**, 125–132.

Dmitriew CM (2011) The evolution of growth trajectories: what limits growth rate? *Biological Reviews*, **86**, 97–116.

Doerge RW, Churchill GA (1996) Permutation tests for multiple loci affecting a quantitative character. *Genetics*, **142**, 285–294.

Duffy GA, Pike TW, Laland KN (2009) Size-dependent directed social learning in nine-spined sticklebacks. *Animal Behaviour*, **78**, 371–375.

Ellegren H, Sheldon BC (2008) Genetic basis of fitness differences in natural populations. *Nature*, **452**, 169–175.

Elphinstone MS, Hinten GN, Anderson MJ, Nock CJ (2003) An inexpensive and high-throughput procedure to extract and purify total genomic DNA for population studies. *Molecular Ecology Notes*, **3**, 317–320.

Flint J, Mackay TFC (2009) Genetic architecture of quantitative traits in mice, flies, and humans. *Genome Research*, **19**, 723–733.

Gjedrem T (2000) Genetic improvement of cold-water fish species. *Aquaculture Research*, **31**, 25–33.

Gonda A, Välimäki K, Herczeg G, Merilä J (2012) Brain development and predation: plastic responses depend on evolutionary history. *Biology Letters*, **8**, 249–252.

Green P, Falls K, Crooks S (1990) *Documentation for CRI-MAP (version 2.4)*. Washington University School of Medicine, St. Louis, Missouri. (http://linkage.rockefeller.edu/soft/crimap/)

Haley CS, Knott SA, Elsen JM (1994) Mapping quantitative trait loci in crosses between outbred lines using least squares. *Genetics*, **136**, 1195–1207.

Heckel G, Zbinden M, Mazzi D *et al.* (2002) Microsatellite markers for the three-spined stickleback (*Gasterosteus aculeatus* L.) and their applicability in a freshwater and an anadromous population. *Conservation Genetics*, **3**, 79–81.

Herczeg G, Gonda A, Merilä J (2009a) Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *Journal of Evolutionary Biology*, **22**, 544–552.

Herczeg G, Gonda A, Merilä J (2009b) Evolution of gigantism in nine-spined stickleback. *Evolution*, **63**, 3190–3200.

Herczeg G, Turtiainen M, Merilä J (2010) Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. *Biological Journal of the Linnean Society*, **101**, 403–416.

Herczeg G, Gonda A, Kuparinen A, Merilä J (2012) Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? *Evolutionary Ecology*, **26**, 109–122.

Hill WG, Kirkpatrick M (2011) What animal breeding has taught us about evolution. *Annual Review of Ecology, Evolution and Systematics*, **41**, 1–19.

Hosoya S, Kai W, Fujita M *et al.* (2012) The genetic architecture of growth rate in juvenile *Takifugu* species. *Evolution*, **67**, 590–598.

Huey RB, Gilchrist GW, Carlson ML, Berrigan D, Serra L (2000) Rapid evolution of a geographic cline in size in an introduced fly. *Science*, **287**, 308–309.

James AC, Azevedo RB, Partridge L (1997) Genetic and environmental responses to temperature of *Drosophila melanogaster* from a latitudinal cline. *Genetics*, **146**, 881–890.

Johnson SL, Gates MA, Johnson M *et al.* (1996) Centromere-linkage analysis and consolidation of the zebrafish genetic map. *Genetics*, **142**, 1277–1288.

Karlsson EK, Lindblad-Toh K (2008) Leader of the pack: gene mapping in dogs and other model organisms. *Nature Reviews Genetics*, **9**, 713–725.

Kocher TD, Lee WJ, Sobolewska H, Penman D, McAndrew B (1998) A genetic linkage map of a cichlid fish, the tilapia (*Oreochromis niloticus*). *Genetics*, **148**, 1225–1232.

Lahti K, Huuskonen H, Laurila A, Piironen J (2002) Metabolic rate and aggressiveness between brown trout populations. *Functional Ecology*, **16**, 167–174.

Laine VN, Primmer CR, Herczeg G, Merilä J, Shikano T (2012) Isolation and characterization of 13 new nine-spined stickleback, *Pungitius pungitius*, microsatellites located nearby candidate genes for behavioural variation. *Annales Zoologici Fennici*, **49**, 123–128.

Lander E, Kruglyak L (1995) Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nature Genetics*, **11**, 241–247.

Largiadèr CR, Fries V, Kobler B, Bakker TCM (1999) Isolation and characterization of microsatellite loci from the three-spined stickleback (*Gasterosteus aculeatus* L.). *Molecular Ecology*, **8**, 342–344.

Laurila A, Pakkasmaa S, Merilä J (2006) Population divergence in growth rate and antipredator defences in *Rana arvalis*. *Oecologia*, **147**, 585–595.

Lee SF, Chen Y, Varan AK *et al.* (2011) Molecular basis of adaptive shift in body size in *Drosophila melanogaster*: functional and sequence analyses of the *Dca* gene. *Molecular Biology and Evolution*, **28**, 2393–2402.

Leinonen T, Cano JM, Mäkinen H, Merilä J (2006) Contrasting patterns of body shape and neutral genetic divergence in marine and lake populations of threespine sticklebacks. *Journal of Evolutionary Biology*, **19**, 1803–1812.

Lieschke GJ, Currie PD (2007) Animal models of human disease: zebrafish swim into view. *Nature Reviews Genetics*, **8**, 353–367.

Lugo JM, Rodriguez A, Helguera Y *et al.* (2008) Recombinant novel pituitary adenylate cyclase-activating polypeptide from African catfish (*Clarias gariepinus*) authenticates its biological function as a growth-promoting factor in low vertebrates. *Journal of Endocrinology*, **197**, 583–597.

Lynch M, Walsh B (1998) *Genetics and Analysis of Quantitative Traits*. Sinauer Associates, Inc., Sunderland, Massachusetts.

Mackay TFC, Stone EA, Ayroles JF (2009) The genetics of quantitative traits: challenges and prospects. *Nature Reviews Genetics*, **10**, 565–577.

Mäkinen HS, Cano JM, Merilä J (2008) Identifying footprints of directional and balancing selection in marine and freshwater three-spined stickleback (*Gasterosteus aculeatus*) populations. *Molecular Ecology*, **17**, 3565–3582.

Merilä J (2013) Nine-spined stickleback (*Pungitius pungitius*): an emerging model for evolutionary biology research. *Annals of the New York Academy of Sciences*, **1289**, 18–35.

Miller CT, Beleza S, Pollen AA *et al.* (2007) cis-Regulatory changes in Kit ligand expression and parallel evolution of pigmentation in sticklebacks and humans. *Cell*, **131**, 1179–1189.

Noor M, Cunningham A, Larkin JC (2001) Consequences of recombination rate variation on quantitative trait locus mapping studies. Simulations based on the *Drosophila melanogaster* genome. *Genetics*, **159**, 581–588.

Outomuro D, Johansson F (2011) The effects of latitude, body size, and sexual selection on wing shape in a damselfly. *Biological Journal of the Linnean Society*, **102**, 263–274.

Peichel CL, Nereng K, Ohgi KA *et al.* (2001) The genetic architecture of divergence between threespine stickleback species. *Nature*, **414**, 901–905.

Podisi BK, Knott SA, Burt DW, Hocking PM (2013) Comparative analysis of quantitative trait loci for body weight, growth rate and growth curve parameters from 3 to 72 weeks of age in female chickens of a broiler-layer cross. *BMC Genetics*, **14**, 22.

Postlethwait JH, Johnson SL, Midson CN *et al.* (1994) A genetic linkage map for the zebrafish. *Science*, **264**, 699–703.
Protas M, Tabansky I, Conrad M *et al.* (2008) Multi-trait evolution in a cave fish, *Astyanax mexicanus*. *Evolution & Development*, **10**, 196–209.
Rebai A, Goffinet B, Mangin B (1995) Comparing power of different methods for QTL detection. *Biometrics*, **51**, 87–99.
Ressler KJ, Mercer KB, Bradley B *et al.* (2011) Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. *Nature*, **470**, 492–497.
Riska B, Atchley WR, Rutledge JJ (1984) A genetic analysis of targeted growth in mice. *Genetics*, **107**, 79–101.
Rocha JL, Eisen EJ, Dale Van Vleck L, Pomp D (2004) A large-sample QTL study in mice: I Growth. *Mammalian Genome*, **15**, 83–99.
Rogers SM, Bernatchez L (2007) The genetic architecture of ecological speciation and the association with signatures of selection in natural lake whitefish (*Coregonus* sp. *Salmonidae*) species pairs. *Molecular Biology and Evolution*, **24**, 1423–1438.
Rohlf FJ (2006) *TPS Software Series*. Distributed by the author. Department of Ecology and Evolution, State University of New York, Stony Brook, New York.
Schütz KE, Kerje S, Jacobsson L *et al.* (2004) Major growth QTLs in fowl are related to fearful behavior: possible genetic links between fear responses and production traits in a red junglefowl x white leghorn intercross. *Behavior Genetics*, **34**, 121–130.
Seaton G, Hernandez J, Grunchec JA *et al.* (2006) GridQTL: A grid portal for QTL mapping of compute intensive datasets. *Proceedings of the 8th World Congress on Genetics Applied to Livestock Production*, August 13-18, 2006. Belo Horizonte, Brazil.
Shapiro MD, Bell MA, Kingsley DM (2006) Parallel genetic origins of pelvic reduction in vertebrates. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 13753–13758.
Shapiro MD, Summers BR, Balabhadra S *et al.* (2009) The genetic architecture of skeletal convergence and sex determination in ninespine sticklebacks. *Current Biology*, **19**, 1140–1145.
Shikano T, Jetty R, Shimada Y, Merilä J (2010a) Utility of sequenced genomes for microsatellite marker development in non-model organisms: a case study of functionally important genes in nine-spined sticklebacks (*Pungitius pungitius*). *BMC Genomics*, **11**, 334.
Shikano T, Ramadevi J, Merilä J (2010b) Identification of local- and habitat-dependent selection: Scanning functionally important genes in nine-spined sticklebacks (*Pungitius pungitius*). *Molecular Biology and Evolution*, **27**, 2775–2789.
Shikano T, Shimada Y, Herczeg G, Merilä J (2010c) History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. *Molecular Ecology*, **19**, 1147–1161.
Shikano T, Natri HM, Shimada Y, Merilä J (2011) High degree of sex chromosome differentiation in stickleback fishes. *BMC Genomics*, **12**, 474.
Shikano T, Laine VN, Herczeg G, Vilkki J, Merilä J (2013) Genetic architecture of parallel pelvic reduction in ninespine sticklebacks. *G3: Genes, Genomics Genetics*, **3**, 1833–1842.
Shimada Y, Shikano T, Kuparinen A, Gonda A, Leinonen T, Merilä J (2011) Quantitative genetics of body size and timing of maturation in two nine-spined stickleback (*Pungitius pungitius*) populations. *PLoS ONE*, **6**, e28859.
Slate J (2005) Quantitative trait locus mapping in natural populations: progress, caveats and future directions. *Molecular Ecology*, **14**, 363–379.
Slate J, Santure AW, Feulner PGD *et al.* (2010) Genome mapping in intensively studied wild vertebrate populations. *Trends in Genetics*, **26**, 275–284.
Studer AJ, Doebley JF (2011) Do large effect QTL fractionate? A case study at the maize domestication QTL *teosinte branched1*. *Genetics*, **188**, 673–681.
Teacher AGF, Shikano T, Karjalainen ME, Merilä J (2011) Phylogeography and genetic structuring of European nine-spined sticklebacks (*Pungitius pungitius*) - mitochondrial DNA evidence. *PLoS ONE*, **6**, e19476.
Trokovic N, Herczeg G, McCairns RJS, Ab Ghani NI, Merilä J (2011) Intraspecific divergence in the lateral line system in the nine-spined stickleback (*Pungitius pungitius*. *Journal of Evolutionary Biology*, **24**, 1546–1558.
Wada H, Naruse K, Shimada A, Shima A (1995) Genetic linkage map of a fish, the Japanese medaka *Oryzias latipes*. *Molecular Marine Biology and Biotechnology*, **4**, 269–274.
Webster MM, Laland KN (2012) Social information, conformity and the opportunity costs paid by foraging fish. *Beavioural Ecology and Sociobiology*, **66**, 797–809.
Wirén A, Wright D, Jensen P (2013) Domestication-related variation in social preferences in chickens is affected by genotype on a growth QTL. *Genes, Brain and Behavior*, **12**, 330–337.
Wright D, Nakamichi R, Krause J, Butlin RK (2006) QTL analysis of behavioral and morphological differentiation between wild and laboratory zebrafish (*Danio rerio*). *Behavior Genetics*, **36**, 271–284.
Xu S (2003) Theoretical basis of the Beavis effect. *Genetics*, **165**, 2259–2268.
Yeaman S, Whitlock MC (2011) The genetic architecture of adaptation under migration-selection balance. *Evolution*, **65**, 1897–1911.
Young WP, Wheeler PA, Coryell VH, Keim P, Thorgaard GH (1998) A detailed linkage map of rainbow trout produced using doubled haploids. *Genetics*, **148**, 839–850.
Zhou H, Deeb N, Evock-Clover CM, Ashwell CM, Lamont SJ (2006) Genome-wide linkage analysis to identify chromosomal regions affecting phenotypic traits in the chicken. I. Growth and average daily gain. *Poultry Science*, **85**, 1700–1711.

This study was conceived by J.M. and G.H. The the data collection was carried out G.H., T.S. and J.M. The data was analysed by V.N.L. with help from J.V. and T.S. The manuscript was written by J.M. and V.N.L., with aid from other authors.

Data accessibility

The input files for GridQTL are deposited at Dryad: doi: 10.5061/dryad.gf7b0.

Appendix I

Linkage groups, locations, marker group (GR, growth; PHYS, physiological; BEH, behavior; RAN, random, see text for details) and related genes of the markers used for linkage map and QTL analyses in this study

Marker	Linkage group	Location (cM)	Group	Related gene
Pun3	LG1	0.0	RAN	
Pun206	LG1	4.0	RAN	
Pun134	LG1	15.7	RAN	
Ppbig13	LG1	27.0	BEH	*SLC6A4*
Umf30	LG1	44.6	RAN	
Pun49	LG1	78.8	RAN	
ATP1A1	LG1	91.7	PHYS	*ATP1A1*
Umf48	LG1	98.5	RAN	
Umf40	LG1	115.1	RAN	
Ppgm29	LG1	117.6	PHYS	*HPX*
Pun145	LG1	121.4	RAN	
Pun251	LG1	121.6	RAN	
Ppgm57	LG1	122.0	PHYS	*TBX4*
Stn439	LG1	122.3	RAN	
Stn438	LG1	124.8	RAN	
Stn437	LG1	136.7	RAN	
Ppbig10	LG1	158.4	BEH	*PGR*
Stn18	LG2	0.0	RAN	
Pprm10	LG2	5.7	RAN	
NHE2b	LG2	36.3	PHYS	*NHE2b*
Pun96	LG2	39.6	RAN	
Umf2	LG2	51.1	RAN	
Umf7	LG2	51.9	RAN	
GAest66	LG2	70.8	RAN	
Ppgm30	LG2	76.7	PHYS	*HSP25*
Stn328	LG3	0.0	RAN	
Pun139	LG3	0.0	RAN	
Pprm1	LG3	0.8	RAN	
Pun205	LG3	6.0	RAN	
ACAPRa	LG3	47.8	GR	*ACAPRa*
Pun188	LG3	52.5	RAN	
Umf15	LG3	62.8	RAN	
Pprm11	LG3	67.9	RAN	
Ppgm7	LG3	69.3	PHYS	*ATP1A2*
Pun227	LG3	70.1	RAN	
Stn433	LG4	0.0	RAN	
Stn432	LG4	19.5	RAN	
Pun175	LG4	22.8	RAN	
FGF18	LG4	23.5	GR	*FGF18*
Ppgm21	LG4	25.8	GR	*FGF18*
GAest35	LG4	37.4	RAN	
Pun109	LG4	43.0	RAN	
Pun334	LG4	45.2	RAN	
Pun316	LG4	47.4	RAN	
Pprm2	LG4	57.9	RAN	
Pun89	LG4	65.6	RAN	
Stn253	LG4	72.9	RAN	
ORSSR253	LG4	78.0	RAN	
Umf16	LG4	81.6	RAN	
Pun95	LG4	97.7	RAN	
Pun272	LG4	121.3	RAN	
Pprm4	LG5	0.0	RAN	
Pun304	LG5	9.3	RAN	
Pun51	LG5	14.2	RAN	
Pun19	LG5	37.1	RAN	
Umf8	LG5	39.5	RAN	
Pun112	LG5	42.8	RAN	
Pun286	LG5	43.7	RAN	
Ppgm42	LG5	75.1	PHYS	*Kir2.2*
Pun178	LG5	80.1	RAN	
Stn434	LG6	0.0	RAN	
Ppbig11	LG6	15.0	BEH	*PKG*
Stn435	LG6	25.1	RAN	
KCNJ12	LG6	37.0	PHYS	*KCNJ12*
Umf49	LG6	64.1	RAN	
Stn436	LG6	68.0	RAN	
Ppgm18	LG6	73.4	GR	*eEF1A1b*
Ppgm10	LG7	0.0	PHYS	*ATP6V1Ab*
Umf33	LG7	4.7	RAN	
Ppbig5	LG7	12.5	BEH	*DRD1*
Ppbig8	LG7	24.4	BEH	*HTR3B*
Stn257	LG7	39.1	RAN	
Pprm12	LG7	54.9	RAN	
Pun319	LG7	57.0	RAN	
Pun78	LG7	57.9	RAN	
Stn89	LG8	0.0	RAN	
Pun184	LG8	1.1	RAN	
Pun68	LG8	8.0	RAN	
Ppgm2	LG8	21.9	GR	*ACAPRb*
Umf3	LG8	28.5	RAN	
GAest7	LG8	29.3	RAN	
Umf6	LG8	30.5	RAN	
Pun207	LG8	39.2	RAN	
Ppgm17	LG8	51.3	PHYS	*DIO1*
Stn100	LG9	0.0	RAN	
Pun238	LG9	14.7	RAN	
Ppgm26	LG9	17.0	PHYS	*GR1*
Pun86	LG9	18.7	RAN	
Umf54	LG9	19.6	RAN	
Stn108	LG9	23.8	RAN	
Ppgm61	LG9	54.5	RAN	
NPY2Rb	LG9	56.2	GR	*NPY2Rb*
Umf71	LG9	59.1	RAN	
Umf47	LG9	66.6	RAN	
Pun257	LG9	79.5	RAN	
GAest14	LG10	0.0	RAN	
Pun144	LG10	20.0	RAN	
Pun221	LG10	24.9	RAN	
Pun156	LG10	27.2	RAN	
Pun309	LG10	30.8	RAN	
Pun42	LG10	44.0	RAN	
Umf9m	LG10	55.3	RAN	
Pun158	LG11	0.0	RAN	
Pun307	LG11	6.6	RAN	

Appendix I *Continued*

Marker	Linkage group	Location (cM)	Group	Related gene
Stn130	LG11	28.7	RAN	
Umf31	LG11	29.8	RAN	
Ppgm51	LG11	30.2	GR	*PVALBb*
Ppgm54	LG11	36.3	GR	*SSR1b*
Stn127	LG11	38.6	RAN	
Ppgm40	LG11	42.6	PHYS	*Kir2.1a*
Umf57	LG11	46.0	RAN	
Pun183	LG11	50.7	RAN	
Pun274	LG11	52.9	RAN	
Pun185	LG11	62.8	RAN	
Pun230	LG11	71.3	RAN	
GS1	LG11	75.1	PHYS	*GS1*
Pun294	LG11	79.6	RAN	
Ppgm14	LG11	86.0	PHYS	*CLCN7*
CLCN7	LG11	86.4	PHYS	*CLCN7*
Pun93	LG11	90.7	RAN	
Pun98	LG12	0.0	RAN	
Pun61	LG12	2.1	RAN	
Pun256	LG12	2.3	RAN	
Ppgm12	LG12	4.7	PHYS	*CLCN3*
Stn71	LG12	32.2	RAN	
Pun110	LG12	52.6	RAN	
Ppsm2	LG12	79.1	RAN	
Pun255	LG12	79.6	RAN	
Stn19	LG12	79.6	RAN	
Ppsm4	LG12	79.6	RAN	
Ppsm5	LG12	80.6	RAN	
Ppsm6	LG12	85.1	RAN	
Pun7	LG12	88.0	RAN	
Ppsm8	LG12	89.7	RAN	
Ppsm9	LG12	93.4	RAN	
Pun2	LG12	95.4	RAN	
Ppgm35	LG12	95.7	PHYS	*HSP70B*
Pun300	LG12	96.0	RAN	
Pun65	LG12	96.4	RAN	
Pun234	LG12	117.3	RAN	
Ppsm13	LG12	118.1	RAN	
Pun192	LG13	0.0	RAN	
Pun163	LG13	9.6	RAN	
Pprm13	LG13	29.7	RAN	
GAest67	LG13	31.1	RAN	
Pun220	LG13	34.9	RAN	
Pun200	LG13	37.8	RAN	
Pun182	LG13	51.9	RAN	
Umf37m	LG13	59.9	RAN	
Pun171	LG13	72.7	RAN	
Pun115	LG13	73.4	RAN	
Pun282	LG13	73.4	RAN	
Pun20	LG13	80.7	RAN	
Pun173	LG13	81.6	RAN	
Pun97	LG13	90.1	RAN	
Pun201	LG13	91.4	RAN	
Umf60	LG13	96.7	RAN	
Ppgm47	LG13	98.5	PHYS	*NKCC1b*
Stn198	LG14	0.0	RAN	
Pun203	LG14	17.0	RAN	

Appendix I *Continued*

Marker	Linkage group	Location (cM)	Group	Related gene
Umf42	LG14	41.5	RAN	
GAest3	LG14	69.4	RAN	
Pun324	LG14	73.9	RAN	
Pun141	LG15	0.0	RAN	
Umf38	LG15	29.6	RAN	
Pun288	LG15	37.5	RAN	
Pun22	LG15	44.9	RAN	
Pun330	LG15	47.1	RAN	
Pprm6	LG15	47.7	RAN	
NHE2c	LG15	50.8	PHYS	*NHE2c*
Stn173	LG15	54.9	RAN	
Pun293	LG15	57.5	RAN	
Pun159	LG15	68.5	RAN	
GAest51	LG16	0.0	RAN	
Pun72	LG16	2.6	RAN	
Stn315	LG16	3.9	RAN	
Pprm7	LG16	11.8	RAN	
Ppbig2	LG16	22.3	BEH	*CCKB*
Pun211	LG16	34.2	RAN	
Umf44	LG16	44.4	RAN	
Pprm14	LG16	47.3	RAN	
Pun180	LG16	72.2	RAN	
Pun210	LG16	74.3	RAN	
Pun261	LG16	95.6	RAN	
Umf10	LG17	0.0	RAN	
Pun301	LG17	5.0	RAN	
Umf63	LG17	10.5	RAN	
Ppgm56	LG17	12.6	PHYS	*TAAR*
Pun52	LG17	12.7	RAN	
Pun212	LG17	13.8	RAN	
Ppgm60	LG17	15.0	RAN	
Pun66	LG17	16.4	RAN	
Pun233	LG17	36.4	RAN	
Pun260	LG18	0.0	RAN	
Stn196	LG18	11.1	RAN	
Umf66	LG18	19.6	RAN	
Pprm8	LG18	25.4	RAN	
Pun153	LG18	27.9	RAN	
Ppbig7	LG18	47.9	BEH	*ESR2*
Stn195	LG18	79.5	RAN	
Umf67m	LG19	0.0	RAN	
Umf65	LG19	1.5	RAN	
Pun130	LG19	16.3	RAN	
Ppgm37	LG19	40.5	PHYS	*HSP90B*
Stn194	LG19	42.3	RAN	
Stn443	LG19	44.6	RAN	
Pun75	LG19	44.6	RAN	
Stn442	LG19	44.6	RAN	
Stn444	LG19	44.6	RAN	
Pun209	LG19	44.9	RAN	
Ppgm20	LG19	52.3	GR	*FGF6a*
Pun117	LG19	66.8	RAN	
IGF-II	LG19	70.9	GR	*IGF-II*
Pun315	LG20	0.0	RAN	
Pun187	LG20	2.6	RAN	
1125PBBE	LG20	6.5	RAN	

Appendix I *Continued*

Marker	Linkage group	Location (cM)	Group	Related gene
Pun162	LG20	37.3	RAN	
7080PBBE	LG20	39.4	RAN	
Ppbig4	LG20	45.4	BEH	*DAT1*
Pun114	LG21	0.0	RAN	
Stn222	LG21	11.3	RAN	

Appendix I *Continued*

Marker	Linkage group	Location (cM)	Group	Related gene
Stn223	LG21	13.6	RAN	
Pun148	LG21	14.8	RAN	
Pun177	LG21	15.9	RAN	
Ppgm9	LG21	23.4	PHYS	*ATP6V1Aa*
Ppgm52	LG21	31.1	PHYS	*SHH*

Behav Genet (2014) 44:77–88
DOI 10.1007/s10519-013-9624-8

ORIGINAL RESEARCH

QTL Analysis of Behavior in Nine-Spined Sticklebacks (*Pungitius pungitius*)

Veronika N. Laine · Gábor Herczeg · Takahito Shikano · Johanna Vilkki · Juha Merilä

Received: 10 May 2013 / Accepted: 12 October 2013 / Published online: 6 November 2013

Abstract The genetic architecture of behavioral traits is yet relatively poorly understood in most non-model organisms. Using an F_2-intercross ($n = 283$ offspring) between behaviorally divergent nine-spined stickleback (*Pungitius pungitius*) populations, we tested for and explored the genetic basis of different behavioral traits with the aid of quantitative trait locus (QTL) analyses based on 226 microsatellite markers. The behaviors were analyzed both separately (viz. feeding activity, risk-taking and exploration) and combined in order to map composite behavioral type. Two significant QTL—explaining on average 6 % of the phenotypic variance—were detected for composite behavioral type on the experiment-wide level, located on linkage groups 3 and 8. In addition, several suggestive QTL located on six other linkage groups were detected on the chromosome-wide level. Apart from providing evidence for the genetic basis of behavioral variation, the results provide a good starting point for finer-scale analyses of genetic factors influencing behavioral variation in the nine-spined stickleback.

Keywords Behavior · Fish · Microsatellite · Personality · QTL · Stickleback

Edited by Stephen Maxson.

V. N. Laine (✉)
Division of Genetics and Physiology, Department of Biology, University of Turku, 20014 Turku, Finland
e-mail: nvlain@utu.fi

G. Herczeg · T. Shikano · J. Merilä
Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, P.O. Box 65, 00014 Helsinki, Finland
e-mail: gaborherczeg@caesar.elte.hu

T. Shikano
e-mail: takahito.shikano@helsinki.fi

J. Merilä
e-mail: juha.merila@helsinki.fi

Present Address:
G. Herczeg
Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/c., Budapest 1117, Hungary

J. Vilkki
MTT Agrifood Research Finland, Jokioinen, Finland
e-mail: johanna.vilkki@mtt.fi

Introduction

Behavioral traits can influence individual fitness through their impact on mate choice, foraging success, predator avoidance and success in competition for resources. Although it has been established that behaviors are often heritable (e.g. Weiss et al. 2000; Bouchard and Loehlin 2001; Dingemanse et al. 2002, 2009; van Oers et al. 2005), the genetic underpinnings of behavioral traits are generally still poorly understood (Boake et al. 2002; van Oers et al. 2005; Tschirren and Bensch 2010). However, recent studies—especially those conducted with humans and other model organisms—have started to uncover the genetic mechanisms underlying different behavioral traits (reviews in: Sokolowski 2001; Fitzpatrick et al. 2005; Inoue-Murayama 2009).

With the help of quantitative trait locus (QTL) mapping it is possible to identify the genomic areas associated with quantitative trait variation (Falconer and Mackay 1996; Lynch and Walsh 1998). However, complex and extremely plastic traits, such as behavior, are challenging to study because individual genes often account for only a small fraction of the measured quantitative variation (Boake et al. 2002; Mackay et al. 2009). Also, compared to most

morphological traits, the heritabilities and repeatabilities of behavioral traits are variable and often relatively low (Mousseau and Roff 1987; Boake 1989; Bell et al. 2009), making the study of their genetic basis even more challenging. Nonetheless, the QTL-mapping approach has successfully identified some behavioral genes of major effect (Flint and Corley 1996; Reif and Lesch 2003; van Oers and Mueller 2010).

Typically, QTL studies in animals have been confined to work conducted with humans, livestock and laboratory strains of model organisms, but recently studies focused on non-model species from the wild have started to emerge (reviewed in Slate 2005; Stinchcombe and Hoekstra 2008). The advantage of using wild populations in QTL studies is that such studies are likely to capture and uncover variation relevant in nature, whereas genetic and phenotypic variation in domesticated and laboratory strains may not be representative of those in the wild (Hoffmann 2002; Slate 2005). Naturally, the drawback in the use of wild-derived individuals is that advanced crossing-schemes—such as inbred F_2 or backcross designs (Lynch and Walsh 1998) which require inbreeding and homozygous lines—are not possible in most cases. Furthermore, studies of outbred populations limited by sample sizes are more prone to overestimating QTL effect sizes which might lead to erroneous inferences (Slate 2013). On the other hand, first or second generation wild-derived individuals are often more genetically variable than laboratory strains, aiding in mapping phenotypic variation in quantitative traits (Falconer and Mackay 1996; Mackay et al. 2009).

Thanks to developments in molecular biology and bioinformatics, it is now easier to conduct QTL studies with wild animals than before (Slate 2005; Ellegren and Sheldon 2008; Slate et al. 2009). An increasing number of QTL-mapping studies have been performed using wild animal strains (e.g. fish: Colosimo et al. 2004; Shapiro et al. 2009; Shikano et al. 2013; mammals: Johnston et al. 2010; birds: Tarka et al. 2010). However, QTL studies of behavioral traits in wild animals are still almost non-existent (but see: Wright et al. 2006a, b; Weber et al. 2013), possibly because of the logistic constraints involved with QTL mapping and the measurement of the behaviors in wild individuals. Recent candidate gene analyses have identified many individual genes linked to behaviors including exploration (Fidler et al. 2007), activity (Takeuchi et al. 2009) and dominance (Burmeister et al. 2007). Moreover, QTL analyses conducted with model organisms have shown that behavioral QTL are distributed across the genome (Wright et al. 2006a, b; Henderson et al. 2004).

The nine-spined stickleback (*Pungitius pungitius*) is a small and widespread fish that is becoming a model for behavioral and evolutionary biology research (Merilä 2013). Fish originating from different environments express contrasting behaviors: individuals from ponds that lack predatory fish exhibit higher feeding activity and are more aggressive, explorative and risk-taking than individuals originating from marine environments (Herczeg et al. 2009b; Herczeg and Välimäki 2011). Although these population differences are suggested to be genetically based as revealed by common garden experiments (Herczeg et al. 2009b, 2013; Herczeg and Välimäki 2011), the genomic underpinnings of these differences remain unknown.

The aim of this study was to identify genomic areas associated with behavioral variation among populations of nine-spined sticklebacks by performing QTL analyses using an F_2 cross based on fish from highly divergent pond and marine populations. To this end, we first constructed a linkage map using a set of microsatellite markers ($n = 226$), and then mapped variation in three behavioral traits (both separately and combined as composite behavioral type sensu Bell 2007) in seven F_2-generation families ($n = 283$) produced by a single pair of fish. We were particularly interested to see if QTL with major effects (e.g. Weber et al. 2013) would be found, or whether the results would conform to the general expectation of several loci each with small effect (e.g. Visscher et al. 2012; Flint and Munafo 2013). In addition, we also expected that pond alleles would be associated with an increase in the feeding activity, risk-taking and explorative behavior of nine-spined stickleback compared to alleles originating from marine populations because of the population differences described above.

Materials and methods

The species and crosses used

The fish forming the grand-parental generation (F_0) were collected in 2006 from a marine population from the Baltic Sea (Helsinki; 60°13′N, 25°11′E) and from a pond population from northeastern Finland (Rytilampi; 66°23′N, 29°19′E). Marine nine-spined sticklebacks belong to a complex fish community with numerous predatory species, whereas pond sticklebacks from Rytilampi are the only fish species occurring in their isolated habitat. Several studies have earlier shown that the fish from marine and pond populations are phenotypically and genetically divergent in their morphology and life-history characteristics (Herczeg et al. 2009a, 2010, 2012; Shimada et al. 2011a; Ab Ghani et al. 2012, 2013; Välimäki and Herczeg 2012; Välimäki et al. 2012), including behavior (Herczeg et al. 2009b, 2013; Herczeg and Välimäki 2011). Pond nine-spined sticklebacks are generally more aggressive, risk-taking, explorative and have higher feeding activity than their marine conspecifics (Herczeg et al. 2009b; Herczeg and Välimäki 2011).

A female fish from the marine population was mated with a male from the pond population and the resulting F_1-offpring was group-reared at ca. 15 °C and 14:10 h (light:dark) photoperiod. After artificial hibernation (6 °C and 0:24 h photoperiod), fish were put gradually into conditions to stimulate reproduction (17 °C and 24:0 h photoperiod). Once the F_1-fish had matured, one randomly selected male and female were placed alone in an aquarium and allowed to mate and produce fertilized clutches. This pair produced seven successive clutches, and the resulting F_2-offspring were placed individually in 1.4 L tanks in zebrafish racks (Aquaneering Inc., San Diego, USA) after hatching. Visibility between the tanks was blocked with white plastic sheets placed between the tanks. Hence, the fish were completely predation- and competition-naïve, and free from parasites that could potentially alter behavior during the rearing period. Racks featured physical, chemical and biological filters. A 14:10 h photoperiod was applied during the whole F_2 rearing period to prevent fish from coming into reproductive condition, which would have altered their behavior. Two weeks after the fish had started to swim freely, digital photographs were taken from each individual and this was repeated every four weeks until they were 187 days old, at which point they were over-anesthetized with MS-222 (tricaine methanesulfonate). The photographing was done for mapping growth strategies (Laine et al. 2013). The behavioral trials (see below) were conducted between the last two periods of photographing, separated by 28 days. Immediately after the last photographs were taken, the fish were over-anesthetized and the fresh weight of each fish was recorded. Landmark-based geometric morphometrics were employed to calculate centroid size, which is the square root of the summed squared distances from the centroid to the different landmarks (Bookstein 1991), and a good proxy for general body size. As we did not analyze body shape in this paper, we do not detail the landmarks here (for landmark positioning see e.g. Herczeg et al. 2010). At the end of the experiment, 283 fish (56, 44, 38, 37, 40, 31 and 27 individuals from the seven clutches, respectively) were available for behavioral trials. Sex was determined by visual inspection of the gonads.

Behavioral measurements

Three behavioral traits were assessed in the following order: feeding activity, risk-taking and exploration. Feeding activity represents behavior in a familiar environment; risk-taking is behavior in a familiar environment after disturbance; and exploration is activity in an unfamiliar environment (see details below). Feeding activity was measured at least one week after the previous photographing to minimize stress effects due to handling. Subsequent behavioral tests of the same individuals were separated by at least four days. Briefly, feeding activity was measured as the time needed for initiating feeding during a normal daily feeding event. Risk-taking was measured as the time needed for initiating feeding after a simulated attack (dropping a shiny metallic bolt [180 mm long, 10 mm diameter] through a hole in the individual container of the focal fish). Exploration was assessed as the time needed to fully leave a refuge in a novel environment (see Herczeg et al. 2009b for more details about these measurements). We note that all behavioral variables are based on latency, hence, high values indicate low behavioral activity and vice versa. For the sake of straightforward interpretation, when we refer to 'high feeding activity', 'high risk-taking' or 'high exploration' hereafter reporting/discussing our results, we mean behavioral activity and not the latency variables *per se*. From the 283 offspring tested, 10 individuals were excluded because of unsuccessful behavioral assays.

The mean dispersion estimates of different behavioral measurements are given in Table 1. Since the different behaviors were intercorrelated (feeding activity—risk-taking: $r_{s(268)} = 0.33$; $P < 0.001$; feeding activity—exploration: $r_{s(268)} = 0.11$, $P = 0.075$; risk-taking—exploration: $r_{s(268)} = 0.17$, $P = 0.005$), we used principal component analysis to collapse them to a smaller set of independent variables. Two principal components (PCs) were extracted: PC1 accounted for 44 % of the total variance and was positively correlated with feeding activity (0.71), risk-taking (0.73) and to a lesser extent, exploration (0.54; Table 1) measurements. Therefore this PC can be viewed as a variable describing the common component of all measured behaviors, i.e. placing the fish along a general shyness-boldness continuum. PC2 accounted for 30 % of the total variance and was strongly positively correlated with exploration (0.83), while feeding activity and risk-taking had only weak and negative loadings on this PC (−0.39 and −0.24, respectively; Table 1). Hence, PC2 can be viewed as a variable describing an independent aspect of exploration. The PCs can be interpreted as independent variables describing variation in composite behavioral type (sensu Bell 2007; for a similar application see Herczeg et al. 2009b; David et al. 2011). However, because behavior is a complex trait, it has been suggested that dividing the "super-character" into smaller "sub-characters" helps to better understand the genetic basis of complex traits (Mather and Jinks 1982; Wright et al. 2006a, b). Therefore we analyzed the individual behaviors (viz. feeding activity, risk-taking and exploration) as well as the composite behavioral types (i.e. the PCs described above). The individual behavioral variables had bimodal distributions and thus violated the assumption of normality. This occurred because some of the individuals moved almost instantly when the trials commenced (low score) whereas others did not move at all during the

Table 1 Number of offspring tested (*n*), mean behavior value, standard deviation (SD) and coefficient of variation (CV) for the original behavior variables and results of the PCA using all three behavioral variables and 268 individuals which had all the measurements

					Loadings	
Trait	*n*	Mean	SD	CV	PC1	PC2
Feeding activity	273	82.21	84.66	102.97	0.71	−0.39
Risk-taking	273	101.28	84.93	83.86	0.73	−0.24
Exploration	268	117.99	119.61	101.37	0.54	0.83
Eigenvalue					1.33	0.83
% variance explained					44	30

Loadings, eigenvalues and the percentage of explained variance for each principal component (PC) are given

observation period (maximum scores). Because showing no activity is actually a type of response to the test situations, and as the ratio of such inactive individuals was habitat-dependent in an earlier study (Herczeg et al. 2009b), these individuals were not excluded from the analyses. Instead, we transformed the original variables into binary variables (active coded as 0 and inactive coded as 1) and mapped them as binary response variables.

DNA extraction and genotyping

Total genomic DNA was extracted from ethanol preserved fins using a silica-fines/microtitre filtration plate method (Elphinstone et al. 2003) following proteinase K digestion. All the F_2-offspring ($n = 283$), their parents ($n = 2$) and grand-parents ($n = 2$) were genotyped for 235 loci which could be divided into three groups based on their location relative to genes with different functions. The groups were (i) gene markers associated with behaviorally relevant genes (Laine et al. 2012a), (ii) gene markers associated with physiologically relevant genes (Shikano et al. 2010, 2011b), and (iii) randomly selected markers (Largiadèr et al. 1999; Peichel et al. 2001; Heckel et al. 2002; Colosimo et al. 2004; Miller et al. 2007; Mäkinen et al. 2008; Shapiro et al. 2009; Shikano et al. 2011). Polymerase chain reactions (PCRs) for all markers except Ppbig were performed in a 10 µl volume containing 1 × Qiagen Multiplex PCR Master Mix (Qiagen, Germany), 0.5 × Q-Solution, 2 pmol of each primer and 10–20 ng of template DNA. One of each primer pair was labeled with FAM, HEX or TET fluorescent dye. PCR cycling started with an initial activation step at 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 90 s and 72 °C for 60 s, and completed with a final extension at 60 °C for 5 min. PCRs for Ppbig markers were conducted according to Laine et al. (2012a). All PCR products were diluted 1:500 with Milli-Q water and genotyped using a MegaBACE 1000 automated sequencer (Amersham Biosciences) with ET-ROX 400 size standard (Amersham Biosciences). Alleles were scored using Fragment Profiler 1.2 program (Amersham Biosciences) and edited by eye. To ensure consistency in genotyping, all alleles were read by the same person.

Fig. 1 Linkage map of the nine-spined stickleback with genetic distances (at *left* of each group) listed in centimorgans. Significant (*bold*; experiment-wide level) and suggestive (chromosome-wide level) QTL found on each linkage group are indicated below the respective linkage groups. See Table 2 for details. *Feed* feeding activity, *Risk* risk-taking

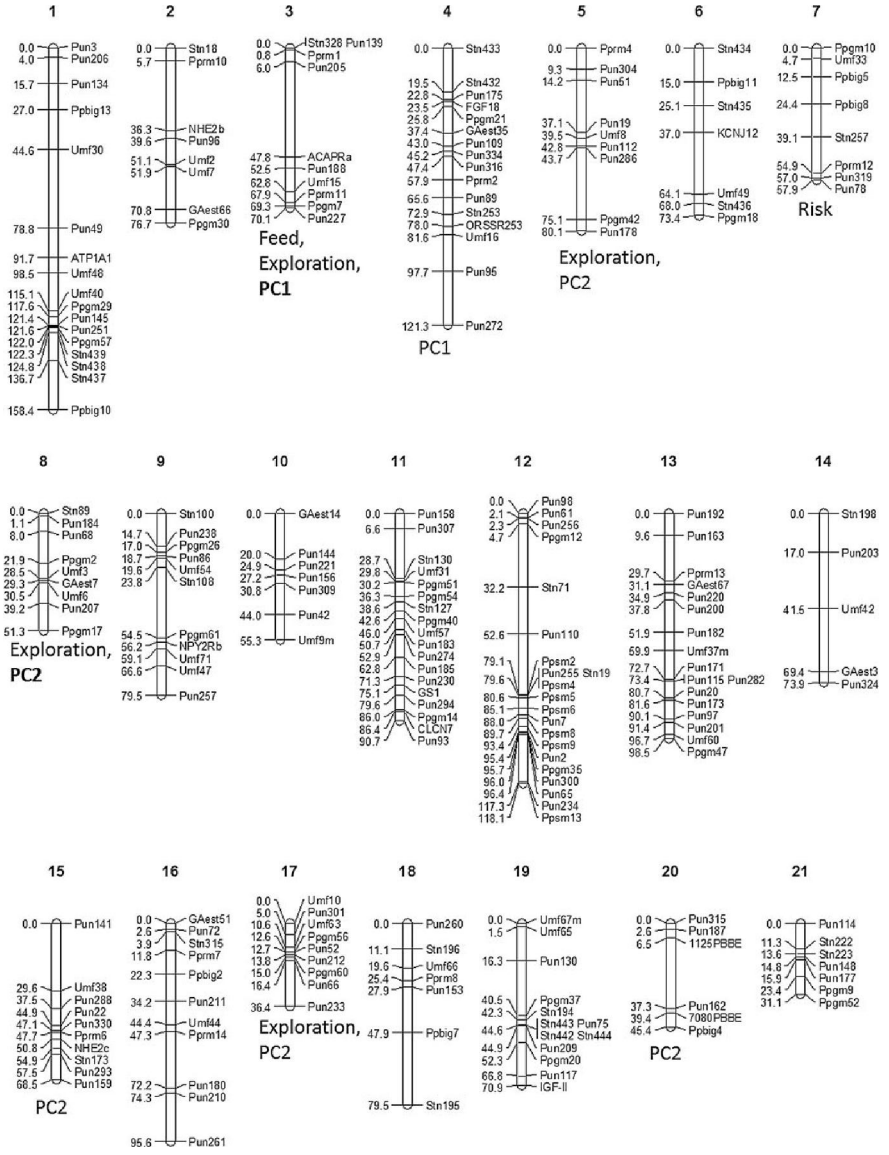
Linkage map

The linkage map used here has been published earlier by Shikano et al. (2013). In short, the map was constructed by using the improved CRI-MAP version 2.5 (Green et al. 1990). The option TWOPOINT was used to get the logarithm of the odds (LOD) score for every pair of markers. A LOD score threshold of three was used as a significant criterion for linkage. In addition, an earlier nine-spined stickleback linkage map (Shapiro et al. 2009) and the three-spined stickleback (*Gasterosteus aculeatus*) genome obtained from Ensembl (genebuild May 2010, database version 66.1) were used as references to help in the initial linkage group forming. To determine the best order of the markers for each linkage group, option BUILD was used by beginning with the most informative marker pair. Markers were fitted manually if they could not be fitted straight with BUILD and with LOD score ≥4.0. The FLIPS option ($n = 3$–5) was used to evaluate the statistical significance of the obtained order. Double recombination events were detected using the CHROMPIC option. Individuals with over four recombinations were removed and a second CRIMAP analysis round was conducted. Final order for each linkage group is presented in Fig. 1.

Initially 235 microsatellite markers were used for linkage mapping but nine of them were discarded in the end due to low polymorphism and low LOD scores in TWOPOINT. Because statistical power in QTL studies is dependent on how informative the marker is (Slate et al. 1999), every marker included into the map had to have over 100 informative meioses (average = 504). Visualization of the map was done by using MAPCHART 2.2 (Voorrips 2002).

QTL mapping

A total of 226 markers were used in the QTL analyses. The genotyping success in offspring varied from 74 to 99 % for each marker. Recombination was sex-biased with the



female recombination rate being approximately twice as high as that in males (Shikano et al. 2013). Accordingly, we used sex-average linkage map distances in QTL mapping. QTL mapping was performed in GridQTL (available at http://www.gridqtl.org.uk/) by using the BCF2 portlet, fitting both additive and dominance effects. Analyses were performed at 1 cM intervals. The percentage of the phenotypic variance explained by the QTL was calculated following Zhou et al. (2006) as:

$$\text{percentage variance } (\%) = ((\text{RRMS} - \text{FRMS})/\text{RRMS}) \times 100, \quad (1)$$

where RRMS is the residual mean square from the reduced model in which all the effects including background QTL effects are fitted but the QTL is left out. The FRMS is the residual mean square from the model in which all the effects and QTL are fitted.

There were no sex differences in mean values of behavioral traits (Fisher's exact test: feeding activity: $P = 0.54$; risk-taking: $P = 0.40$; exploration: $P = 0.26$; t test: PC1: $t_{266} = 0.41$, $P = 0.68$; PC2: $t_{266} = 1.53$, $P = 0.13$). Significant correlations were found between centroid size and the following variables: feeding activity (t_{271} −2.27 $P = 0.023$), risk-taking ($t_{245.50} = -2.81$, $P = 0.005$), PC1 ($r_{268} = -0.20$, $P = 0.001$), and PC2 ($r_{268} = 0.16$, $P = 0.011$). Therefore, centroid size was included as a covariate in all QTL-analyses except that concerned with exploration. In addition, significant differences between clutches were found in every behavioral variable except PC1 (feeding activity $F_{6,\ 266} = 3.87$, $P = 0.001$; risk-taking $F_{6,\ 266} = 3.49$, $P = 0.003$; exploration $F_{6,\ 261} = 2.20$, $P = 0.043$; PC1 $F_{6,\ 261} = 1.16$, $P = 0.146$; PC2 $F_{6,\ 261} = 2.59$, $P = 0.019$) thus, clutch information was added as fixed effect in QTL-analyses.

To evaluate the significance of detected QTL on chromosome- and experiment-wide levels, permutation tests were performed using 10000 iterations (Churchill and Doerge 1994; Doerge and Churchill 1996). QTL were considered significant when the F-value was above the 5 % experiment-wide threshold and suggestive when it was above the 5 % chromosome-wide threshold (Lander and Kruglyak 1995; Kukekova et al. 2011). Confidence intervals were obtained with bootstrap analysis with 10,000 iterations.

All the primary data behind this publication has been uploaded to Dryad respiratory (doi:10.5061/dryad.62380).

Results

QTL results

The sex-averaged linkage map spanned 1,632.7 cM with an average intermarker distance of 7.2 cM. Altogether eight significant (experiment-wide) or suggestive (chromosome-wide) areas in the linkage map showed association with the measured behavioral traits (Table 2; Fig. 1), and the variance explained by these individual QTL varied from 3 to 7 % (Table 2). More additive effects were observed compared to dominance effects (Table 2).

The significant composite behavioral type QTL were located on linkage groups (LGs) 3 (PC1) and 8 (PC2). In addition, feeding activity and exploration were associated with LG 3 but the location of the QTL differed from that of PC1 (Table 2). The association on LG 8 was also found for exploration in same location. The highest LOD score was found for PC1 on LG 3, and also the F-value exceeded the genome-wide 0.01 threshold (Table 2). In addition, a suggestive QTL for risk-taking was detected on LG 7 at the same location where the pituitary homeobox transcription factor 1 (*Pitx1*) is located (marker Pun319).

None of the microsatellite markers associated with behaviorally relevant genes coincided with the identified QTL "peak" locations. However, in many cases, the 95 % confidence interval covered a large area of the linkage groups which included areas where markers for behaviorally and physiologically relevant genes resided (Table 2; Fig. 1).

Discussion

We discovered two significant and six suggestive QTL regions associated with behaviors that have been shown to be highly divergent among pond and marine nine-spined stickleback populations (e.g. Herczeg et al. 2009b). Together these QTL accounted for 4–20 % of the total phenotypic variance in each of the behavioral variables. The strongest association—explaining 7 % of the variation—was found for the principal component (PC1) describing the common component of all measured behavioral traits (i.e. positioning the individuals along a general shyness–boldness continuum), located on LG 3. Another significant QTL was found on LG 8 for the other principal component (PC2) describing exploration independent from the other behaviors. These two QTL are good candidate areas for follow up studies for the identification of causal genetic factors. In what follows, we discuss these results and their implications for our understanding of the genetic basis of behavioral traits in sticklebacks.

In addition to the composite behavioral type QTL (PC1) on LG 3, we found other suggestive QTL influencing feeding activity and explorative behavior on LG 3, and risk taking on LG 7. Suggestive QTL for exploration were also found on LGs 5, 8 and 17.The exploration QTL on these LGs co-located with the PC2 QTL. Similar QTL locations between traits may suggest that the genetic factors

dc_892_14

Table 2 Estimates of linkage group (LG) location, 95 % confidence intervals (CI), test statistic (F), the logarithm of odds (LOD) score, chromosome and experiment wide thresholds for both 1 and 5 % significance levels, additive and dominance effects (±SE) (positive score indicates that the allele from the marine population increases the trait value i.e. more non-moving fish) and percentage of variation explained (PVE) for behavior related QTL

Trait	LG	Location (cM)	CI	F	LOD	Chromosome wide (0.01)	Chromosome wide (0.05)	Experiment wide (0.01)	Experiment wide (0.05)	Additive effect (SE)	Dominance effect (SE)	PVE	Total PVE/trait	Associated candidate gene marker
Feeding activity														
Risk-taking	3	0	0–63	6.79	2.87	6.74	4.96	10.33	8.54	0.13 (0.04)	−0.11 (0.06)	4	4	ACAPRa
	7	57	3–57	7.22	3.05	6.67	4.84	10.22	8.45	−0.09 (0.04)	0.18 (0.06)	4	4	Ppbig5/DRD1: Ppbig8/HTR3B
Exploration														
	3	25	0–70	6.43	2.73	6.93	4.89	10.44	8.50	0.12 (0.05)	−0.24 (0.09)	4		ACAPRa; Ppgm7/ ATP1A2
	5	43	0–75	5.67	2.41	6.85	4.94			−0.13 (0.04)	−0.08 (0.05)	3		
	8	38	2–48	6.04	2.56	6.50	4.73			−0.13 (0.04)	0.01 (0.05)	4		Ppgm2/ACAPRb
	17	0	0–36	5.23	2.23	6.44	4.51			−0.11 (0.04)	−0.08 (0.05)	3	14	
PC1														
	3	6	0–63	**10.92**	4.56	6.89	4.94	10.51	8.56	0.30 (0.08)	−0.34 (0.12)	7		ACAPRa
	4	45	0–99	7.35	3.11	7.46	5.53			0.34 (0.09)	−0.11 (0.13)	5	12	Ppgm21/FGF18
PC2														
	5	43	14–68	5.49	2.34	6.68	4.95	10.26	8.50	−0.30 (0.09)	−0.04 (0.12)	3		
	8	35	1–41	**9.46**	3.97	6.80	4.94			−0.37 (0.09)	−0.02 (0.13)	6		Ppgm2/ACAPRb
	15	11	0–68	5.26	2.24	6.70	4.85			0.33 (0.12)	−0.30 (0.19)	3		NHE2c
	17	0	0–17	7.34	3.10	6.33	4.41			−0.26 (0.08)	−0.26 (0.12)	5		Ppgm56/TAAR
	20	2	0–44	5.12	2.18	6.44	4.54			0.21 (0.09)	0.23 (0.12)	3	20	

F-values in bold are significant at experiment-wide level and the rest of the values are suggestive QTL (chromosome-wide significance)

influencing one behavior may have pleiotropic effects on other behaviors, or that the genetic factors influencing different behaviors cluster onto the same linkage group. The resolution of our linkage map does not allow us to differentiate between these alternatives. In those cases where the QTL positions clearly differ between traits, it can be deduced that different genetic factors underlie different behaviors, such as on LG 3 the QTL for exploration and feeding activity/PC1. When there is a correlation between measured behaviors, the correlated characters can be associated mostly with same linkage groups. This was demonstrated in a behavioral QTL study done with zebrafish (*Danio rerio*) where boldness was divided into subcharacters (Wright et al. 2006b). However, in an extensive anxiety-related behavior QTL study done with mice (*Mus musculus*), it was found that different types of anxiety were associated with different parts of the genome (Henderson et al. 2004). Hence, it seems that in our study the two measured main behavioral components, PC1 and PC2, were different from each other to map to different parts of nine-spined stickleback genome whereas the correlated traits (exploration and PC2) mapped to similar locations.

In many cases, the observed QTL-effects matched our a priori expectations. For instance, marine alleles decreased feeding activity and risk-taking. However, there were also cases where alleles from the pond population decreased behavioral activity in contrast to our predictions, for example, in exploration on LGs 5, 8 and 17. Similar effects were also seen in PC2 on same LGs which is logical as PC2 was mostly correlated with exploration. For example, for the PC2 QTL on LG 8, the genotypes of the F_1-individuals (with "+" indicating a pond allele, and "−" indicating a marine allele) are: F_1 female ∓ and F_1 male ∓. All F_2 offspring with a pond allele, either heterozygous or homozygous, had a higher PC2 value indicating more non-moving fish. In an earlier study it has been shown that high level of heterozygosity in random microsatellite markers is associated with boldness in nine-spined sticklebacks suggesting overall heterozygosity being important to fitness related traits such as behavior (Laine et al. 2012b, see below). One of the hypothesis in the heterozygosity-fitness correlation studies is the general effect hypothesis which predicts that individual heterozygosity reflects genome-wide heterozygosity and that this is expected to be correlated with the individual's inbreeding coefficient and, thus, to be associated with fitness (Hansson & Westerberg 2002). Furthermore, it has been proposed that random and supposedly neutral loci may reflect variation across the genome and thus represent the level of inbreeding depression (Laine et al. 2012b). High heterozygosity has been linked for example in salmonids with aggression, competitive performance, dominance rank and predator avoidance (Tiira et al. 2003, 2006; Vilhunen et al. 2008; Blanchet et al. 2009). Variation (heterozygosity) in the wild has been probably maintained at the QTL loci affecting exploration, therefore these loci are not behaving according to the expectation (that the loci affecting the characteristic traits of the grandparental populations would be fixed to different alleles in the populations). Due to this variation, alleles with "cryptic" effects are found in our study because the QTL genotypes of the picked grandparents are not known. Other explanation for these differing effects could be the new allele combinations in the offspring. Because different behaviors are commonly results of many genes acting together, combining the alleles from two populations in the offspring might lead to different behaviors than in grandparents because of the interactions between the new alleles.

An extensive review about behavior QTL studies conducted with mice and rats (*Rattus norvegicus*) noted that many QTL exhibited small effect sizes (Flint 2003). The fact that each of our discovered QTL explained 3–7 % of the phenotypic variance in behavioral traits aligns with this trend (e.g. Flint 2003; Henderson et al. 2004; Schitz et al. 2004; Wright et al. 2006a, b; see also: Flint and Munafo 2013 for recent review). The power of our study to detect QTL explaining 10 % of phenotypic variation is almost 1.0 and the power to detect QTL explaining 2 % is over 0.4 (Rebai et al. 1995). Therefore, there is only a low chance that we did not detect QTL of substantially larger effect than those we have identified. Rather, QTLs with small effect were quite likely to be detected. However, when considering the probable Beavis effect (Beavis 1994, 1998; theoretically tested in Xu 2003), the detected QTL effect sizes are likely to be biased upward. The confidence intervals for QTL positions were large and in most cases they covered the whole linkage group. In a simulation study, Beavis (1994,1998) showed that the average estimates of phenotypic variances associated with correctly identified QTL were greatly overestimated if only 100 progeny were evaluated, yet the accuracy improved from slightly overestimated to fairly close to the actual magnitude if 500 or 1,000 progeny were evaluated, respectively. Therefore adding more individuals would have increased the power of our experiment and in addition, more markers would have improved the resolution of the QTL, possibly dividing larger QTL areas into smaller linked QTL with lower effect size due to increased accuracy (Mackay et al. 2009). Also, we note that due to logistic constraints, we could not assess the behaviors repeatedly, and thus we could not establish the repeatability of the different traits. Considering that the average repeatability of behavior is often in the range of 0.37–0.47 depending on the approach used to estimate it (Bell et al. 2009), stronger signals might have been found if the individuals had been repeatedly measured and the means of repeated measures had been used in mapping.

Two out of eight microsatellites associated with genes of behavioral relevance (Laine et al. 2012a) were found to map within the QTL confidence interval regions. The remaining six behavioral candidate genes might also have an effect on the studied traits, but relevant variation might not have existed in these genes in our mapping population. The associated behavioral gene-related microsatellites at the risk-taking QTL on LG7 were Ppbig5 and Ppbig8 that are linked with genes dopamine receptor D_1 (*DRD1*) and serotonin receptor 3B (*HTR3B*), respectively. Genomic variations in dopamine receptors (five human dopamine receptor subtypes, D_1, D_2, D_3, D_4 and D_5) are often associated with behavioral differences in various species (Noblett and Coccaro 2005). Allelic variation in subtype D_1 (gene *DRD1*) has been associated with aggression in dogs (Våge et al. 2010), reproductive behavior in chicken (Xu et al. 2010) and attention-deficit/hyperactivity disorder and autism in humans (Misener et al. 2004; Hettinger et al. 2008). In addition to the dopaminergic pathway, many of the studied genes in behavioral genetics also come from the serotonergic pathway. Serotonin regulates many biological processes, and it is commonly involved in anxiety, impulsivity, and aggression-related behaviors (Roth 1994; Berger et al. 2009). Gene *HTR3B* is a candidate gene for antisocial behavior in humans (Ducci et al. 2009). Both of these genes could be important shaping the nine-spined stickleback behavior. However, to confirm their role, further studies are needed.

When inspecting the markers associated with physiologically relevant traits (Shikano et al. 2010; Shimada et al. 2011b), we found several QTL close to genes related to growth (pituitary adenylate cyclase-activating polypeptide type I receptor a [ACAPRa/*ADCYAP1R1a*]) on LG 3, Ppgm21 (fibroblast growth factor 18 [*FGF18*]) on LG 4, Ppgm2 (pituitary adenylate cyclase-activating polypeptide type I receptor b [*ADCYAP1R1b*]) on LG 8), osmoregulation (Ppgm7 (Na^+/K^+-ATPase alpha-subunit isoform 2 [*ATP1A2*]) on LG 3, ATP-sensitive inward rectifier potassium channel 12 [*KCNJ12*] on LG6, sodium/hydrogen exchanger [*NHE2c*] on LG 15), and taste (Ppgm56 (amine-associated receptor [*TAAR*]) on LG 17). In the above mentioned heterozygosity-behavior correlation study (Laine et al. 2012b), heterozygosities in gene markers associated with physiologically relevant genes were correlated with boldness (similar to our exploration variable) and behavioral type (similar to our PC1 representing the general shyness–boldness continuum). More specifically, osmoregulation-related genetic markers were strongly associated with aggression and behavioral type. These relationships were only seen in the marine (Helsinki) population where the fish are exposed not only to high predation risk but also to variable salinity levels in the wild. These results suggest genetic coupling between physiological processes and behavior. However, whether these occur due to pleiotropy or linkage remains to be investigated.

We did not find any major genes affecting behavior. Instead we detected many QTL with small or intermediate effects. This was expected based on other behavior QTL studies (see above). Previous QTL studies done with nine- and three-spined sticklebacks, concentrating primarily on the parallel evolution of e.g. armor plate and pelvis reductions, have found major QTL for these traits. In three-spined sticklebacks armor plate-related traits have been mapped to LG 4 [gene *Ectodysplasin* (*Eda*)] and pelvic traits to LG 7 (gene *Pituitary homeobox transcription factor 1* (*Pitx1*)) (Cole et al. 2003; Colosimo et al. 2004, 2005; Cresko et al. 2004; Shapiro et al. 2004; Coyle et al. 2007; Chan et al. 2010). Interestingly, when comparing Northern European nine-spined sticklebacks to North American populations, pelvic reduction was mapped to different locations (Europe: LG 7; North America: LG 4), indicating independent evolution of the genetic background between different nine-spined stickleback lineages (Shapiro et al. 2009; Shikano et al. 2013). One of the surprising results found from our behavior QTL study is the association between risk-taking and LG 7. This QTL situated in the same location (57 cM, marker Pun319) where gene for pelvic reduction was mapped in European nine-spined sticklebacks (Shikano et al. 2013). This is also the same LG on which the QTL for pelvic reduction is located in three-spined sticklebacks (Cole et al. 2003; Cresko et al. 2004; Shapiro et al. 2004; Coyle et al. 2007). In addition to affecting the pelvis structure in stickleback fishes, a recent candidate gene study in humans has showed that *Pitx1* is associated with behavioral aspects of autism such as failure in stress/anxiety control (Philippi et al. 2007). *Pitx1* is a regulatory factor for the expression of hormones of the pituitary-hypothalamic-adrenal (or interrenal in fishes) axis (HPA) which is known to be involved in stress responses (Lamonerie et al. 1996). In addition to mammals, it has also been shown in fish that HPA activity is regulated by the serotonergic system (Winberg et al. 1997; Bell et al. 2007) which is an important factor in behavioral processes (Berger et al. 2009). Thus, in addition to genes influencing serotonergic system, our results hint about the possibility that *Pitx1* might be influencing fish behavior.

Conclusions

The information about the genetic architecture of behavioral traits in wild populations is still scarce. This study uncovered several behavioral QTL in the nine-spined stickleback genome, each with small to moderate effect sizes. The results further suggest that the genetic

architecture differs between measured traits. In some cases cryptic inheritance was discovered where alleles from marine population increased explorative behavior. Possible candidate genes were also found for future studies to identify genetic factors associated with observed behaviors.

Acknowledgments We thank Abigel Gonda, Mirva Turtianen and Pirkko Siikamäki (the Oulanka Research Station; University of Oulu) for help in obtaining the fish, Kirsi Kähkönen for help in genotyping, Jacquelin De Faveri for comments that improved an earlier version of this manuscript and DJ de Koning for suggestions regarding treating of the trait data. Our research was supported by Academy of Finland (grant numbers 34728, 250435 and 265211to J.M. and 128716 to G.H.) and the Biological Interactions Graduate School (to V.N.L). G.H. was also supported by the Hungarian Scientific Research Fund (G.H. # OTKA-K 105517) and the János Bólyai Research Scholarship of the Hungarian Academy of Sciences. The experiments carried out in this study were conducted under license from the Finnish National Animal Experiment Board (license no. STH211A), and the fish from Kuusamo were obtained with license from Metsähallitus (permit given for G.H.).

References

Ab Ghani NI, Herczeg G, Merilä J (2012) Body size divergence in nine-spined sticklebacks: disentangling additive genetic and maternal effects. Biol J Linn Soc 107:521–528

Ab Ghani NI, Herczeg G, Leinonen T, Merilä J (2013) Evidence for genetic differentiation in timing of maturation among nine-spined stickleback populations. J Evol Biol 26:775–782

Beavis WD (1994) The power and deceit of QTL experiments: lessons from comparative QTL studies. In: Proceedings of the Forty-Ninth Annual Corn, Sorghum Industry Research Conference. pp. 250–266

Beavis WD (1998) QTL analyses: power, precision, and accuracy. In: Paterson AH (ed) Molecular dissection of complex traits. CRC Press, New York, pp 145–162

Bell AM (2007) Future directions in behavioural syndromes research. Proc R Soc Lond B 274:755–761

Bell AM, Backström T, Huntingford FA, Pottinger TG, Winberg S (2007) Variable neuroendocrine responses to ecologically-relevant challenges in sticklebacks. Physiol Behav 91:15–25

Bell AM, Hankison SJ, Laskowski KL (2009) The repeatability of behaviour: a meta-analysis. Anim Behav 77:771–783

Berger M, Gray JA, Roth BL (2009) The expanded biology of serotonin. Annu Rev Med 60:355–366

Blanchet S, Bernatchez L, Dodson JJ (2009) Does interspecific competition influence relationships between heterozygosity and fitness-related behaviors in juvenile Atlantic salmon (*Salmo salar*)? Behav Ecol Sociobiol 63:605–615

Boake CRB (1989) Repeatability: its role in evolutionary studies of mating behavior. Evol Ecol 3:173–182

Boake CRB, Arnold SJ, Breden F, Meffert LM, Ritchie MG, Taylor BJ, Wolf JB, Moore AJ (2002) Genetic tools for studying adaptation and the evolution of behavior. Am Nat 160:S143–S159

Bookstein FL (1991) Morphometric tools for landmark data: geometry and biology. Cambridge University Press, Cambridge

Bouchard TJ, Loehlin JC (2001) Genes, evolution, and personality. Behav Genet 31:243–273

Burmeister SS, Kailasanath V, Fernald RD (2007) Social dominance regulates androgen and estrogen receptor gene expression. Horm Behav 51:164–170

Chan YF, Marks ME, Jones FC et al (2010) Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a *Pitx1* enhancer. Science 327:302–305

Churchill GA, Doerge RW (1994) Empirical threshold values for quantitative trait mapping. Genetics 138:963–971

Cole NJ, Tanaka M, Prescott A, Tickle CA (2003) Expression of limb initiation genes and clues to the basis of morphological diversification in threespine sticklebacks. Curr Biol 13:R951–R952

Colosimo PF, Peichel CL, Nereng K, Blackman BK, Shapiro MD, Schluter D, Kingsley DM (2004) The genetic architecture of parallel armor plate reduction in threespine sticklebacks. PLoS Biol 2:635–641

Coyle SM, Huntingford FA, Peichel CL (2007) Parallel evolution of *Pitx1* underlies pelvic reduction in Scottish threespine stickleback (*Gasterosteus aculeatus*). J Hered 98:581–586

Cresko WA, Amores A, Wilson C, Murphy J, Currey M, Phillips P, Bell MA, Kimmel CB, Postlethwait JH (2004) Parallel genetic basis for repeated evolution of armor loss in Alaskan threespine stickleback populations. Proc Natl Acad Sci USA 101:6050–6055

David M, Auclair Y, Cézilly F (2011) Personality predicts social dominance in female zebra finches, *Taeniopygia guttata*, in a feeding context. Anim Behav 81:219–224

Dingemanse NJ, Both C, Drent PJ, van Oers K, van Noordwijk AJ (2002) Repeatability and heritability of exploratory behaviour in great tits from the wild. Anim Behav 64:929–938

Dingemanse NJ, Van der Plas F, Wright J, Réale D, Schrama M, Roff DA, Van der Zee E, Barber I (2009) Individual experience and evolutionary history of predation affect expression of heritable variation in fish personality and morphology. Proc R Soc Lond B 276:1285–1293

Doerge RW, Churchill GA (1996) Permutation tests for multiple loci affecting a quantitative character. Genetics 142:285–294

Ducci F, Enoch M-A, Yuan Q, Shen PH, White KV, Hodgkinson C, Albaugh B, Virkkunen M, Goldman D (2009) *HTR3B* is associated with alcoholism with antisocial behavior and alpha EEG power—an intermediate phenotype for alcoholism and co-morbid behaviors. Alcohol 43:73–84

Ellegren H, Sheldon BC (2008) Genetic basis of fitness differences in natural populations. Nature 452:169–175

Elphinstone MS, Hinten GN, Anderson MJ, Nock CJ (2003) An inexpensive and high-throughput procedure to extract and purify total genomic DNA for population studies. Mol Ecol Notes 3:317–320

Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics. Longman, New York

Fidler AE, van Oers K, Drent PJ, Kuhn S, Mueller JC, Kempenaers B (2007) *Drd4* gene polymorphisms are associated with personality variation in a passerine bird. Proc R Soc Lond B 274:1685–1691

Fitzpatrick MJ, Ben-Shahar Y, Smid HM, Vet LEM, Robinson GE, Sokolowski MB (2005) Candidate genes for behavioural ecology. Trends Ecol Evol 20:96–104

Flint J, Corley R (1996) Do animal models have a place in the genetic analysis of quantitative human behavioural traits? J Mol Med 74:515–521

Flint J, Munafo M (2013) Herit-ability. Science 340:1416–1417

Green P, Falls K, Crooks S (1990) Documentation for CRI-MAP (version 2.4). Washington University School of Medicine, St. Louis, Missouri. http://linkage.rockefeller.edu/soft/crimap/

Heckel G, Zbinden M, Mazzi D, Kohler A, Reckeweg G, Bakker TCM, Largiadér CR (2002) Microsatellite markers for the three-spined stickleback (*Gasterosteus aculeatus L.*) and their applicability in a freshwater and an anadromous population. Conserv Genet 3:79–81

Henderson ND, Turri MG, DeFries JC, Flint J (2004) QTL analysis of multiple behavioral measures of anxiety in mice. Behav Genet 34:267–293

Herczeg G, Välimäki K (2011) Intraspecific variation in behaviour: effects of evolutionary history, ontogenetic experience and sex. J Evol Biol 24:2434–2444

Herczeg G, Gonda A, Merilä J (2009a) Evolution of gigantism in nine-spined sticklebacks. Evolution 63:3190–3200

Herczeg G, Gonda A, Merilä J (2009b) Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). J Evol Biol 22:544–552

Herczeg G, Turtiainen M, Merilä J (2010) Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. Biol J Linn Soc 101:403–416

Herczeg G, Gonda A, Kuparinen A, Merilä J (2012) Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? Evol Ecol 26:109–122

Herczeg G, Ab Ghani NI, Merilä J (2013) Evolution of stickleback feeding behaviour: genetics of population divergence at different ontogenetic stages. J Evol Biol 26:955–962

Hettinger JA, Liu X, Schwartz CE, Michaelis RC, Holden JJA (2008) A *DRD1* haplotype is associated with risk for autism spectrum disorders in male-only affected sib-pair families. Am J Med Genet Part B 147B:628–636

Hoffmann AA (2002) Laboratory and field heritabilites: some lessons from *Drosophila*. In: Mousseau TA, Sinervo B, Endler JA (eds) Adaptive genetic variation in the wild. Oxford University Press, Oxford, pp 200–218

Inoue-Murayama M (2009) Genetic polymorphism as a background of animal behavior. Anim Sci J 80:113–120

Johnston SE, Beraldi D, McRae AF, Pemberton JM, Slate J (2010) Horn type and horn length genes map to the same chromosomal region in soay sheep. Heredity 104:196–205

Kukekova AV, Trut LN, Chase K, Kharlamova AV, Johnson JL, Temnykh SV, Oskina IN, Gulevich RG, Vladimirova AV, Klebanov S, Shepeleva DV, Shikhevich SG, Acland GM, Lark KG (2011) Mapping loci for fox domestication: deconstruction/reconstruction of a behavioral phenotype. Behav Genet 41:593–606

Laine VN, Herczeg G, Shikano T, Primmer CR (2012a) Heterozygosity-behaviour correlations in nine-spined stickleback (*Pungitius pungitius*) populations: contrasting effects at random and functional loci. Mol Ecol 21:4872–4884

Laine VN, Primmer CR, Herczeg G, Merilä J, Shikano T (2012b) Isolation and characterization of 13 new nine-spined stickleback, *Pungitius pungitius*, microsatellites located nearby candidate genes for behavioural variation. Ann Zool Fenn 49:123–128

Laine VN, Shikano T, Herczeg G, Vilkki J, Merilä J (2013) Quantitative trait loci for growth and body size in the nine-spined stickleback *Pungitius pungitius* L. Mol Ecol. doi:10.1111/mec.12526

Lamonerie T, Tremblay JJ, Lanctôt C, Therrien M, Gauthier Y, Drouin J (1996) Ptx1, a bicoid-related homeo box transcription factor involved in transcription of the pro-opiomelanocortin gene. Genes Dev 10:1284–1295

Lander E, Kruglyak L (1995) Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet 11:241–247

Largiadèr CR, Fries V, Kobler B, Bakker TCM (1999) Isolation and characterization of microsatellite loci from the three-spined stickleback (*Gasterosteus aculeatus L.*). Mol Ecol 8:342–344

Lynch M, Walsh B (1998) Genetics and Analysis of Quantitative Traits, Sinauer Associates

Mackay TFC, Stone EA, Ayroles JF (2009) The genetics of quantitative traits: challenges and prospects. Nat Rev Genet 10:565–577

Mäkinen HS, Cano JM, Merilä J (2008) Identifying footprints of directional and balancing selection in marine and freshwater three-spined stickleback (*Gasterosteus aculeatus*) populations. Mol Ecol 17:3565–3582

Mather K, Jinks JL (1982) Biometrical genetics. The study of continuous variation. Chapman and Hall, London

Merilä J (2013) Nine-spined stickleback (*Pungitius pungitius*): an emerging model for evolutionary biology research. Ann N Y Acad Sci 1289:18–35

Miller CT, Beleza S, Pollen AA, Schluter D, Kittles RA, Shriver MD, Kingsley DM (2007) *cis*-Regulatory changes in kit ligand expression and parallel evolution of pigmentation in sticklebacks and humans. Cell 131:1179–1189

Misener VL, Luca P, Azeke O, Crosbie J, Waldman I, Tannock R, Roberts W, Malone M, Schachar R, Ickowicz A, Kennedy JL, Barr CL (2004) Linkage of the dopamine receptor D1 gene to attention-deficit/hyperactivity disorder. Mol Psychiatry 9:500–509

Mousseau TA, Roff DA (1987) Natural selection and the heritability of fitness components. Heredity 59:181–197

Noblett KL, Coccaro EF (2005) Molecular genetics of personality. Curr Psychiatry Rep 7:73–80

Peichel CL, Nereng K, Ohgi KA, Cole BLE, Colosimo PF, Buerkle CA, Schluter D, Kingsley DM (2001) The genetic architecture of divergence between threespine stickleback species. Nature 414:901–905

Philippi A, Tores F, Carayol J, Rousseau F, Letexier M, Roschmann E, Lindenbaum P, Benajjou A, Fontaine K, Vazart C, Gesnouin P, Brooks P, Hager J (2007) Association of autism with polymorphisms in the paired-like homeodomain transcription factor 1 (PITX1) on chromosome 5q31: a candidate gene analysis. BMC Med Genet 8:74

Rebai A, Goffinet B, Mangin B (1995) Comparing power of different methods for QTL detection. Biometrics 51:87–99

Reif A, Lesch K-P (2003) Toward a molecular architecture of personality. Behav Brain Res 139:1–20

Roth B (1994) Multiple serotonin receptors: clinical and experimental aspects. Ann Clin Psychiatry 6:67–78

Schütz KE, Kerje S, Jacobsson L, Forkman B, Carlborg O, Andersson L, Jensen P (2004) Major growth QTLs in fowl are related to fearful behavior: possible genetic links between fear responses and production traits in a red junglefowl x white leghorn intercross. Behav Genet 34:121–130

Shapiro MD, Marks ME, Peichel CL, Blackman BK, Nereng KS, Jónsson B, Schluter D, Kingsley DM (2004) Genetic and developmental basis of evolutionary pelvic reduction in three-spine sticklebacks. Nature 428:717–723

Shapiro MD, Summers BR, Balabhadra S, Aldenhoven JT, Miller AL, Cunningham CB, Bell MA, Kingsley DM (2009) The genetic architecture of skeletal convergence and sex determination in ninespine sticklebacks. Curr Biol 19:1140–1145

Shikano T, Ramadevi J, Shimada Y, Merilä J (2010) Utility of sequenced genomes for microsatellite marker development in non-model organisms: a case study of functionally important genes in nine-spined sticklebacks (*Pungitius pungitius*). BMC Genomics 11:334

Shikano T, Natri HM, Shimada Y, Merilä J (2011) High degree of sex chromosome differentiation in stickleback fishes. BMC Genomics 12:474

Shikano T, Laine VN, Herczeg G, Vilkki J, Merilä J (2013) Genetic architecture of parallel pelvic reduction in ninespine sticklebacks. G3: Genes, Genomics. Genetics. 3:1833–1842

Shimada Y, Shikano T, Kuparinen A, Gonda A, Leinonen T, Merilä J (2011a) Quantitative genetics of body size and timing of maturation in two nine-spined stickleback (*Pungitius pungitius*) populations. PLoS One 6:e28859

Shimada Y, Shikano T, Merilä J (2011b) A high incidence of selection on physiologically important genes in the three-spined stickleback, *Gasterosteus aculeatus*. Mol Biol Evol 28:181–193

Slate J (2005) Quantitative trait locus mapping in natural populations: progress, caveats and future directions. Mol Ecol 14:363–379

Slate J (2013) From beavis to beak colour: a simulation study to examine how much QTL mapping can reveal about the genetic architecture of quantitative traits. Evolution 67:1251–1262

Slate J, Pemberton JM, Visscher PM (1999) Power to detect QTL in a free-living polygynous population. Heredity 83:327–336

Slate J, Gratten J, Beraldi D, Stapley J, Hale M, Pemberton JM (2009) Gene mapping in the wild with SNPs: guidelines and future directions. Genetica 136:97–107

Sokolowski MB (2001) *Drosophila*: genetics meets behaviour. Nat Rev Genet 2:879–890

Stinchcombe JR, Hoekstra HE (2008) Combining population genomics and quantitative genetics: finding the genes underlying ecologically important traits. Heredity 100:158–170

Takeuchi Y, Hashizume C, Arata S, Inoue-Murayama M, Maki T, Hart BL, Mori Y (2009) An approach to canine behavioural genetics employing guide dogs for the blind. Anim Genet 40:217–224

Tarka M, Akesson M, Beraldi D, Hernández-Sánchez J, Hasselquist D, Bensch S, Hansson B (2010) A strong quantitative trait locus for wing length on chromosome 2 in a wild population of great reed warblers. Proc R Soc Lond B 277:2361–2369

Tiira K, Laurila A, Peuhkuri N, Piironen J, Ranta E, Primmer CR (2003) Aggressiveness is associated with genetic diversity in landlocked salmon (*Salmo salar*). Mol Ecol 12:2399–2407

Tiira K, Laurila A, Enberg K et al (2006) Do dominants have higher heterozygosity? Social status and genetic variation in brown trout, *Salmo trutta*. Behav Ecol Sociobiol 59:657–665

Tschirren B, Bensch S (2010) Genetics of personalities: no simple answers for complex traits. Mol Ecol 19:624–626

Våge J, Wade C, Biagi T, Fatjó J, Amat M, Lindblad-Toh K, Lingaas F (2010) Association of dopamine- and serotonin-related genes with canine aggression. Genes Brain Behav 9:372–378

Välimäki K, Herczeg G (2012) Ontogenetic and evolutionary effects of predation and competition on nine-spined stickleback (*Pungitius pungitius*) body size. J Anim Ecol 81:859–867

Välimäki K, Herczeg G, Merilä J (2012) Morphological anti-predator defences in the nine-spined sticklebacks: constitutive, induced or both? Biol J Linn Soc 107:854–866

Van Oers K, Mueller JC (2010) Evolutionary genomics of animal personality. Proc R Soc Lond B 365:3991–4000

van Oers K, de Jong G, van Noordwijk A, Drent P (2005) Contribution of genetics to the study of animal personalities: a review of case studies. Behaviour 142:1185–1206

Vilhunen S, Tiira K, Laurila A, Hirvonen H (2008) The bold and the variable: fish with high heterozygosity act recklessly in the vicinity of predators. Ethology 114:7–15

Visscher PM, Goddard ME, Derks EM, Wray NR (2012) Evidence-based psychiatric genetics, AKA the false dichotomy between common and rare variant hypotheses. Mol Psychiatry 17:474–485

Voorrips RE (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. J Hered 93:77–78

Weber JN, Peterson BK, Hoekstra HE (2013) Discrete genetic modules are responsible for complex burrow evolution in *Peromyscus* mice. Nature 493:402–405

Weiss A, King JE, Figueredo AJ (2000) The heritability of personality factors in chimpanzees (*Pan troglodytes*). Behav Genet 30:213–221

Winberg S, Nilsson A, Hylland P, Söderström V, Nilsson GE (1997) Serotonin as a regulator of hypothalamic-pituitary-interrenal activity in teleost fish. Neurosci Lett 230:113–116

Wright D, Butlin RK, Carlborg O (2006a) Epistatic regulation of behavioural and morphological traits in the zebrafish (*Danio rerio*). Behav Genet 36:914–922

Wright D, Nakamichi R, Krause J, Butlin RK (2006b) QTL analysis of behavioral and morphological differentiation between wild and laboratory zebrafish (*Danio rerio*). Behav Genet 36:271–284

Xu S (2003) Theoretical basis of the Beavis effect. Genetics 165:2259–2268

Xu H, Shen X, Zhou M, Fang M, Zeng H, Nie Q, Zhang X (2010) The genetic effects of the dopamine D1 receptor gene on chicken egg production and broodiness traits. BMC Genet 11:17

Zhou H, Deeb N, Evock-Clover CM, Ashwell CM, Lamont SJ (2006) Genome-wide linkage analysis to identify chromosomal regions affecting phenotypic traits in the chicken. I. Growth and average daily gain. Poult Sci 85:1700–1711

dc_892_14

The Journal of Experimental Biology 215, 2760-2773

doi:10.1242/jeb.068122

RESEARCH ARTICLE

Spectral tuning by selective chromophore uptake in rods and cones of eight populations of nine-spined stickleback (*Pungitius pungitius*)

Pia Saarinen*,†, Johan Pahlberg*,‡, Gábor Herczeg, Martta Viljanen, Marika Karjalainen, Takahito Shikano, Juha Merilä and Kristian Donner

Department of Biosciences, University of Helsinki, P.O. Box 65, FI-00014 Helsinki, Finland

*These authors contributed equally to this work
†Author for correspondence (pia.k.saarinen@helsinki.fi)
‡Present address: Department of Physiology and Biophysics, Zilkha Neurogenetic Institute, University of Southern California, Los Angeles, CA 90089, USA

SUMMARY

The visual pigments of rods and cones were studied in eight Fennoscandian populations of nine-spined stickleback (*Pungitius pungitius*). The wavelength of maximum absorbance of the rod pigment (λ_{max}) varied between populations from 504 to 530 nm. Gene sequencing showed that the rod opsins of all populations were identical in amino acid composition, implying that the differences were due to varying proportions of chromophores A1 and A2. Four spectral classes of cones were found (two S-cones, M-cones and L-cones), correlating with the four classes of vertebrate cone pigments. For quantitative estimation of chromophore proportions, we considered mainly rods and M-cones. In four populations, spectra of both photoreceptor types indicated A2 dominance (population mean λ_{max}=525–530 nm for rods and 535–544 nm for M-cones). In the four remaining populations, however, rod spectra (mean λ_{max}=504–511 nm) indicated strong A1 dominance, whereas M-cone spectra (mean λ_{max}=519-534 nm) suggested substantial fractions of A2. Quantitative analysis of spectra by three methods confirmed that rods and cones in these populations use significantly different chromophore proportions. The outcome is a shift of M-cone spectra towards longer wavelengths and a better match to the photic environment (light spectra peaking >560 nm in all the habitats) than would result from the chromophore proportions of the rods. Chromophore content was also observed to vary partly independently in M- and L-cones with potential consequences for colour discrimination. This is the first demonstration that selective processing of chromophore in rods and cones, and in different cone types, may be ecologically relevant.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/??/????/DC1

Key words: rhodopsin, porphyropsin, photoreceptor, visual ecology.

Received 11 November 2011; Accepted 10 April 2012

INTRODUCTION

The absorptance spectrum of a visual pigment describes how efficiently it catches photons of different energies (light of different wavelengths) to initiate vision. For pigments serving vision near absolute threshold (rod pigments in most vertebrates), optimal properties can be defined rather simply: the pigment should maximize the signal-to-noise ratio (SNR) by absorbing efficiently the light wavelengths (photon energies) available in the environment, but at the same time be thermally stable to minimize spontaneous activations, i.e. noise (Barlow 1956; Baylor et al., 1980). Because spectral and thermal properties are interdependent (Barlow 1957; Ala-Laurila et al., 2004a; Ala-Laurila et al., 2004b; Luo et al., 2011), optimal positioning of the absorbance spectrum will often imply a trade-off between these two demands. The spectral positioning of cone visual pigments, however, is often best considered in relation to wavelength discrimination rather than absolute sensitivity (Barlow 1958; Vorobyev and Osorio, 1998; Vorobyev et al., 2001a; Vorobyev et al., 2001b). Cones generally operate in brighter light where photon fluctuations constitute the most powerful source of pigment-initiated noise, hence thermal stability of the visual pigment may be less crucial.

All visual pigments are G-protein coupled receptors with a light-catching cofactor (the chromophore) covalently bound to the protein (opsin). Spectral absorbance and thermal stability depend on the interaction of the opsin and the chromophore, and may be tuned by modifying either component (Bridges, 1972; Hargrave et al., 1983; Nathans, 1990a; Nathans, 1990b; Yokoyama and Yokoyama, 2000). Evolutionary adaptation to different light regimes by opsin-based tuning (fixation of new mutations by natural selection) is a fairly slow process, typically requiring at least 10^3–10^4 generations (Terai et al., 2006; Jokela-Määttä et al., 2005; Jokela-Määttä et al., 2009; Larmuseau et al., 2009; Larmuseau et al., 2010). Admittedly, in some fishes, differential expression of multiple genes in the same photoreceptors, e.g. during different stages of life history, provides a mechanism for 'fast' opsin-dependent tuning of spectral sensitivity (Shand 1993; Browman and Hawryshyn 1994; Archer et al., 1995; Hope et al., 1998; Carleton and Kocher 2001; Shand et al., 2002; Spady et al., 2005; Spady et al., 2006; Parry et al., 2005).

Chromophore-based tuning in vertebrates implies changing proportions of two alternative chromophores, A1 (11-*cis* retinal) and A2 (11-*cis* 3,4-didehydroretinal), an option available to fish, amphibians and some reptiles (see Bridges, 1972). The capacity for

chromophore-based tuning is genetically determined (requiring conversion of A1 into A2 by a retinol dehydrogenase), but when present, it allows tuning pigments on a physiological time scale, e.g. seasonally (Beatty, 1975; Temple et al., 2006) or for different stages of life history (Wald, 1946; Carlisle and Denton, 1959; Reuter, 1969; Reuter et al., 1971). The A1 to A2 switch red-shifts and broadens the absorption spectrum (Dartnall and Lythgoe, 1965; Whitmore and Bowmaker, 1989; Hárosi, 1994; Govardovskii et al., 2000; Parry and Bowmaker, 2000; see also Bridges, 1972) by lowering the energy barrier for activation, and is always associated with a decrease in thermal stability of the pigment (Ala-Laurila et al., 2004a; Ala-Laurila et al., 2004b; Ala-Laurila et al., 2007; Luo et al., 2008; Luo et al., 2011).

The Baltic and Fennoscandian region provides a 'natural laboratory' for the study of visual evolution in aquatic animals, offering multiple populations of several species that have become isolated in water bodies with different spectral properties following the retreat of Pleistocene ice sheets during the past ~9000 years (Donner, 1995; Eronen et al., 2001). Here, we have studied to what extent and by what mechanisms the visual pigments of nine-spined sticklebacks (*Pungitius pungitius*) have diverged spectrally in five freshwater and three marine populations. The questions we addressed were: (1) are there differences in the rod opsin that might underlie differential tuning of the rod visual pigment; (2) do sticklebacks use chromophore-based tuning of the visual pigments and, if so, are there consistent differences between populations in this respect; and (3) if rod and/or cone visual pigments differ between populations, can the differences be functionally interpreted as enhancing some aspects of visual performance in the illumination conditions now prevailing in their respective habitats?

MATERIALS AND METHODS

Animals

Nine-spined sticklebacks [*Pungitius pungitius* (Linnaeus 1758)] from eight Fennoscandian populations were used, four of which were Finnish (Rytilampi, Pyöreälampi, Iso-Porontima and Helsinki), two Swedish (Abborrtjärn and Bölesviken) and two Russian (Levin Navolok and Mashinnoye; see Fig. 1). Henceforth, we shall denote the populations by three-letter abbreviations as shown on the map. Three of the habitats (HEL, BOL and WHI) were brackish or saltwater environments, whereas five were freshwater ponds (RYT, PYO, ABB and MAS) or lakes (ISO). Most of these populations have been reproductively isolated for the past 8000 years or so (Eronen et al., 2001), except for MAS, which has been separated from WHI for less than a century. On the basis of microsatellite data, the MAS population is indistinguishable from WHI, whereas those of all other ponds and lakes are very different from any other population (Shikano et al., 2010). The sea and freshwater populations, including MAS, already show distinct morphological, behavioural, neuroanatomical and life history adaptations, presumably chiefly in response to reduced predatory pressure in isolated ponds lacking predatory fish (Gonda et al., 2011; Herczeg et al., 2009a; Herczeg et al., 2009b; Herczeg and Välimäki, 2011; Trokovic et al., 2011).

After capture in June, the fish were transferred in tanks to the animal care facilities at the University of Helsinki, kept in aerated freshwater aquaria at approximately 15°C under a 12 h:12 h light:dark cycle (in a windowless room with fluorescent tubes) and provided with appropriate food. All fish studied by microspectrophotometry (MSP) were kept in these constant conditions for more than 6 months before recordings were carried out in the following year (see below). The experiments were

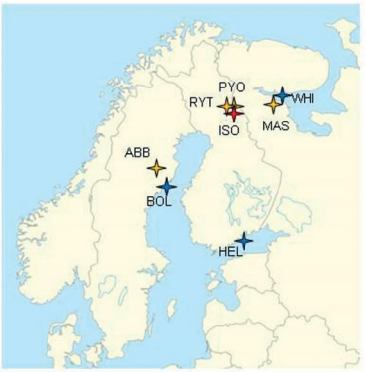


Fig. 1. Geographic locations of the habitats of the eight populations studied: Abborrtjärn (ABB), Bölesviken (BOL), Helsinki (HEL), Iso-Porontima (ISO), Mashinnoye (MAS), Pyöreälampi (PYO), Rytilampi (RYT) and White Sea at Levin Navolok (WHI). Sea habitats are marked by blue stars, ponds by yellow stars and the lake by a red star.

authorized by the Animal Experiment Board at the University of Helsinki and the National Animal Experiment Board of Finland.

DNA analyses

The gene coding for the protein (opsin) part of the rod visual pigment was sequenced to see whether there was any variation in coding sequences. Total genomic DNA of three individuals from each of the eight populations was extracted using NaOH boiling (Duan and Fuerst, 2001). Teleost primers were kindly provided by Prof. David Hunt (Lions Eye Institute, University of Western Australia) and Dr Wayne Davies (Nuffield Laboratory of Ophthalmology, Oxford University), designed for nested PCR to amplify the fragments containing the transmembrane part of the rod opsin gene: 5′-ACAGAGGGACCCTHYTTYTAYRTCCCYATG-3′ RH1_F1, 5′-CATCCTBVTBGGHTTYCCRTCAACTTCCT-3′ RH1_F2, 5′-CTTCCCRCAGCACADKGTGGTGAKCATGCA-3′ RH1_R1 and 5′-GCTGGARGASRCDGADGARGCCTCGGTCTT-3′ RH1_R2. The primer order is 5′…F1…F2…R1…R2…3′ so the possible primer combinations and amplicon sizes are as follows: F1–R2, 1038 bp; F1–R1, 966 bp; F2–R1, 835 bp; and F2–R2, 907 bp.

Initial PCR consisted of 1× Phire Reaction Buffer [20 mmol l^{-1} Tris-HCl, 0.1 mmol l^{-1} EDTA, 1 mmol l^{-1} DTT, 100 mmol l^{-1} KCl, stabilizers, 200 μg ml^{-1} bovine serum albumin (BSA) and 50% glycerol; ThermoFisher Scientific, Waltham, MA, USA], 0.2 mmol l^{-1} of each dNTP (ThermoFisher Scientific), 5 pmol of primers F1 and R2, 0.2 μl of Phire Hot Start I DNA Polymerase (ThermoFisher Scientific) and approximately 10 ng of DNA in the final reaction volume of 10 μl. The temperature profile consisted of preliminary denaturation at 98°C for 30 s followed by 30 cycles of 98°C for 10 s, 48°C for 10 s, 72°C for 30 s and final extension at 72°C for 1 min. Nested PCR was conducted with 1:20 diluted F1+R2 amplicon as a template with the same protocols (exception: 25 cycles instead of 30) for primer pairs F1–R1, F2–R1 and F2–R2. PCR products were then incubated for 30 min at 37°C with 5 U of Antarctic Phosphatase (New England Biolabs, Ipswich, MA, USA), 50 U of Exonuclease I (New

England Biolabs), 1× Antarctic Phosphatase Buffer and 1× Exonuclease I Buffer. Cycle sequencing reactions were run with the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Fragments were cleaned with the Montage SEQ96 Sequencing Reaction Cleanup Kit (Merck Millipore, Billerica, MA, USA) before running them in capillary electrophoresis (MegaBACE, GE Healthcare, Piscataway, NJ, USA). Sequences were aligned using MEGA 5 (Tamura et al., 2011) and deposited in GenBank (accession numbers JQ619637–JQ619638).

Microspectrophotometry

Absorbance spectra of visual pigments were recorded by MSP in single outer segments of rods and cones. All fish used for MSP were adult individuals of average length (4–6 cm). Koli (Koli, 1990) reports that the typical length of sexually mature Finnish nine-spined sticklebacks is 3–5 cm, but fish from ponds are generally somewhat bigger than fish from the sea. Recordings were obtained in 2008 in all eight populations: in February–April for ABB, ISO, PYO, RYT, BOL and HEL, and in August–October for WHI and MAS (data from 47 individuals). In 2011, additional samples from the populations HEL, PYO and RYT were studied in January–June (data from 22 individuals); further, two more RYT individuals were studied in autumn 2011. The numbers of individuals (*N*) studied in each population in the two years, and the months of recording, are given in Table 1. The replications in 2011 served two purposes. Firstly, it was essential to check how constant the properties were of each population, especially the differences in chromophore ratios between populations, over two different years for fish that had been treated similarly and studied at roughly the same time of the year. Secondly, preparation procedures during 2008 were carried out under dim red light (λ>650 nm). Although this light will have negligible effect on rods and on short- and middle-wavelength-sensitive cones (S- and M-cones, respectively), it will bleach the visual pigment of long-wavelength-sensitive cones (L-cones) to varying extent. In 2011, all procedures were carried out under infrared light (LED with peak emission at 850 nm and 50 nm half-bandwidth) with the aid of an infrared viewer.

Table 1. Wavelengths of peak absorbance and chromophore content in rods and M-cones in eight populations of nine-spined stickleback

Data set	Population and recording season (*N*)	Photoreceptor type (*n*)	Template λ_{max} (±s.e.m.)	A1 (%)	PCA component peak	Percent explained
1	Abbortjärn / ABB (6) 2/2008	Rod (107)	507.8±0.6	97.6	501.8	38.3
		Cone (126)	519.2±0.7	70.6	516.8	58.1
2	Iso-porontima / ISO (5) 2/2008	Rod (137)	511.0±1.4	84.6	503.5	57
					571.4	2.7
		Cone (75)	523.3±1.7	60.5	515.8	59.1
					580.6	2.4
3	Pyöreälampi / PYO (6) 4/2008	Rod (108)	504.8±0.4	92.5	504.5	60.0
		Cone (59)	518.6±0.6	68.4	526.2	60.0
					515.2	7.6
4	Pyöreälampi / PYO (7/6) 2–5/2011	Rod (225)	507.9 ±0.6	96.2	507.2	65.9
					574.2	1.0
		Cone (131)	534.3±1.8	39.7	532.5	77.0
					519.8	7.1
					546.8	4.9
					589.5	2.6
5	Rytilampi / RYT (6) 2–3/2008	Rod (180)	507.9±1.5	90.1	500.8	60.0
					560.8	2.3
		Cone (108)	520.3±1.0	73.1	517.4	66.9
6	Bölesviken / BOL (7/6) 2/2008	Rod (153)	527.6±1.3	19.6	523,0	41.3
		Cone (92)	541.7±1.6	4.1	535.5	45.6
7	Helsinki / HEL (5) 3/2008	Rod (98)	527.3±1.4	5.4	–	–
		Cone (78)	538.0±2.7	35.5	–	–
8	Helsinki / HEL (8/6) 1–5/2011	Rod (251)	529.0±0.6	11.2	525.2	79.0
		Cone (242)	544.8±2.3	23.1	530.5	66.9
					618.5	1.1
9	White Sea / WHI (5/3) 4–10/2008	Rod (112)	528.6±1.4	50.8	536.2	54.2
					569.9	5.6
		Cone (31)	536.0±1.1	12.4	523.8	35.1
					536.3	16.3
10	Mashinnoye / MAS (6) 8–9/2008	Rod (145)	529.5±0.6	7.3	525.4	45.6
		Cone (95)	543.5±1.0	3.1	535.1	34.3
11	Rytilampi / RYT (6) 3–5/2011	Rod (148)	516.5±3.2	62.7	513.5	66.8
					569.2	2.1
		Cone (212)	540.6±1.6	36.2	534.2	61.6
					603.5	2.1

The populations are divided into two groups according to chromophore dominance in rods: group 1 (data sets 1–5), with A1-dominated rods; and group 2 (data sets 6–0), with A2-dominated rods.
See Results for comments on data set 9 (WHI), and data set 11 (RYT).
N, number of individuals included; *n*, total number of cells from which spectra are included; λ_{max}, Govardovskii et al. (Govardovskii et al., 2000) parameter obtained by fitting sums of A1 and A2 templates to the full spectrum of each individual; A1 (%), percentage of A1 calculated as the mean of estimates obtained by the two independent methods 1 and 2 (see Materials and methods).
Results of principal component analysis (PCA) are shown as the peak wavelengths of major PCA components, and percent of the total variance explained by each of them (method 3).

Before experiments, the fish to be used was kept in complete darkness for at least 2 h (usually longer, for up to 24 h). The fish was then decapitated and the brain and spinal cord were destroyed with a preparation needle. The pelvic fins were cut off and stored in absolute ethanol for later genomic analyses. The eyes were enucleated and one eye was stored in formalin for morphological analyses. The other eye was hemisected in teleost Ringer's solution (110 mmol l^{-1} NaCl, 2.5 mmol l^{-1} KCl, 1 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgSO_4$, 10 mmol l^{-1} $NaHCO_3$, 10 mmol l^{-1} glucose and 10 mmol l^{-1} HEPES, pH≈7.5). The central part of the isolated retina (detached from the pigment epithelium) was transferred to a drop of Ringer's solution on a microscope slide and teased apart to isolate photoreceptors and expose their outer segments. Dextran (relative molecular mass=66,900) was added to the Ringer's solution to immobilize cells during measurements. The sample was covered with a coverslip, sealed at the edges with Vaseline to prevent drying, and put on the MSP stage.

Absorbance spectra were recorded with a single-beam, computer-controlled, fast wavelength-scanning microspectrophotometer built at the University of Helsinki according to the basic design of Govardovskii and Zueva (Govardovskii and Zueva, 1988; Govardovskii and Zueva, 2000). The recordings were made at room temperature from single outer segments of rods and cones, which could be distinguished morphologically. Each cell was measured only once. The size of the measuring beam was adjusted to fit the outer segment, typically the whole length and ¾ of the width, although the full width was used in small cone outer segments to maximize SNR. The beam was linearly polarized in the plane of the discs. The wavelength calibration was checked at the beginning and end of each experiment against the spectrum of a 'blue glass' standard (Schott BG20, Mainz, Germany), which had been accurately measured in a Hitachi spectrophotometer. The data were stored on a computer hard disk and the basic processing was as described by Govardovskii et al. (Govardovskii et al., 2000). Raw spectra from single cells were averaged and normalized for each individual separately, and in case there was obvious zero offset and/or drift, the spectra were corrected by fitting a new baseline through points at long wavelengths, where absorbance was negligible, >650 or >700 nm depending on cell type. (This was not possible in A2-dominated L-cones.) Further technical details can be found in Govardovskii et al. and Ala-Laurila et al. (Govardovskii et al., 2000; Ala-Laurila et al., 2002).

Our main analysis is focussed on rods and M-cones (see below). Recordings from both classes of photoreceptors were obtained from 69 individuals. Rods were very abundant and on average 20–30 good rod spectra were collected from each individual. The total number of spectra from single rods included in the analyses is 1761. The M-cone spectra obtained from the same individuals were also averaged from recordings of 20–30 cells each, with a total of 1489 single-cell spectra included in the analyses. S-cones were much less abundant and had smaller outer segments. Therefore, S-cone spectra were obtained from only 55 of the 69 individuals, typically averaged from five to 10 cells in each (359 S-cones in total). Sampling of L-cone spectra in 2008 was unrepresentative, as especially the A2 version of the visual pigment was bleached by the red preparation light (see above). In 2011, when only infrared light was used, spectra were obtained from 210 L-cones with population mean λ_{max} ranging from 551 to 602 nm, and only these were used for quantitative analysis.

Analysis of spectra

Even if only a single opsin is present, analysis of spectra of an A1:A2 mixture entails determination of two unknown variables, λ_{max} of either component and the A1:A2 proportion. In the following, we generally express the former variable in terms of λ_{max}(A1), whereby λ_{max}(A2) is fixed by Hárosi's (Hárosi, 1994) formula for the general relation first described by Dartnall and Lythgoe (Dartnall and Lythgoe, 1965), hereafter termed the DLH relationship, and the latter as A1 percentage, A1(%). For a theoretical, noise-free spectrum, it would be possible to find a unique solution by fitting sums of standard A1 and A2 pigment templates, relying on the fact that the component spectra differ in shape (see Govardovskii et al., 2000). For noisy spectra, however, fits of approximately equal quality may be achieved with different value pairs [λ_{max}(A1), A1(%)]. Within certain limits, increasing one and decreasing the other have rather similar effects on the shape of the mixed spectrum. When applied to recorded spectra, where different portions may be affected by quite different types of noise, the ambiguity cannot be resolved by any statistical procedures. Disambiguation by prior assumptions, in contrast, will introduce systematic error. Our strategy was therefore to use two essentially independent methods that rely on different subsets of the spectral data, and are not sensitive to the same sources of systematic error. Our final conclusions rely on the consistency of results obtained by these two methods. As a third method providing additional insight, we applied principal component analysis (PCA).

Method 1: template fitting and determination of chromophore content based on the shape of spectra

Sums of A1 and A2 templates of Govardovskii et al. (Govardovskii et al., 2000) in varying proportions were fitted to the α-band of normalized, averaged and zero-line corrected spectra of each individual (see above). Fitting implies setting the two parameters λ_{max}(A1) and A1(%) by iterative toggling until finding a best fit, as judged by eye on the computer screen. It is important to note that method 1 relies almost entirely on the sloping parts of spectra and is insensitive to data points around the peak (see Fig. 2). Moreover, it makes no prior assumptions on λ_{max}(A1) or λ_{max}(A2). These two properties make it independent of method 2, described next.

Method 2: determination of chromophore content from the wavelength of peak absorbance

The wavelength of peak absorbance of a mixture of A1 and A2 pigments based on the same opsin is fully defined by the proportions of the two components and their λ_{max}. If either λ_{max}(A1) or λ_{max}(A2) is known (mutually coupled by the DLH relationship), A1(%) can be calculated from the λ_{max} of the mixture. To make method 2 formally independent of method 1, we did not use the λ_{max} value obtained by template fitting, but used an estimate based exclusively on data around the peak. These estimates (here denoted λ_{maxp}) were obtained by least-square fitting of second-order polynomials (parabolas) to data points ±30 nm and ±50 nm around the template-based λ_{max}, which was used only as a provisional anchoring point (cf. Ala-Laurila et al., 2002). The two vertex values (λ_{v30} and λ_{v50}) thus obtained were averaged to give $\lambda_{maxp}=(\lambda_{v30}+\lambda_{v50})/2$. Estimating A1(%) requires that λ_{max}(A1) or λ_{max}(A2) be fixed by some independent assumption. As there is no way of knowing how close any single measurement may be to a 'pure' component, fixing these end points is the main source of potential systematic error of method 2. For rods, the lower and upper bounds of population mean λ_{max} were 504.8 and 529.5 nm, respectively. If these represented pure A1 and pure A2, respectively, the complementary values according to the DLH relationship would be λ_{max}(A2)=531.5 nm and λ_{max}(A1)=503.3 nm. Thus the observed range is sufficiently close to the DLH prediction to warrant the conclusion that the observed

doi:10.1242/jeb.062893

population boundaries in rods correspond to nearly pure A1 and A2. For M-cones, however, the population extremes were 519.0 and 543.5 nm, defining a range much narrower than predicted from their respective DLH complements λ_{max}(A2)=555 nm and λ_{max}(A1)=512.5 nm. Thus, only one of the observed boundaries (at best) can correspond to pure A1 or pure A2. Because the highest value, 543.5 nm, was found in two highly A2-dominated populations (MAS and HEL), and other A2-dominated populations were close to that (see Results), we fixed M-cone λ_{max}(A2) at 543.5 nm, giving λ_{max}(A1)=512.5 nm.

Fig. 2 illustrates the difference between methods 1 and 2. Consider the M-cone data (green). The template fit to the full spectrum in panel A was achieved with a sum of 30% A1 and 70% A2. The λ_{max} values of the components are unrealistic (and not used for any further analysis); they emerge only as collaterals to the characterization of the shape of the spectrum by a sum of DLH-coupled template pairs without prior constraints on λ_{max}(A1) or λ_{max}(A2). Most importantly, the data points around the actual peak of the spectrum play no role in template-fitting other than defining the amplitude of the spectrum. In Fig. 2A, this is illustrated by the two other curves (violet, pure A1 template; orange, pure A2 template), which have been set to have the same λ_{max} (543.4 nm) and peak value (here normalized to unity) as the best-fitting template, yet fail conspicuously in reproducing most of the spectrum. By contrast, method 2 is defined to take into account only the data points around peak. Fig. 2B illustrates least-square parabola fits to data ±30 nm around peak of the same recordings as in panel A. This gives the value 547 nm for the cone peak, not so different from the 543.4 nm of the template fit. (The correlation of λ_{max} values as determined by the two types of fits for all individuals is shown in Fig. 4A,B.)

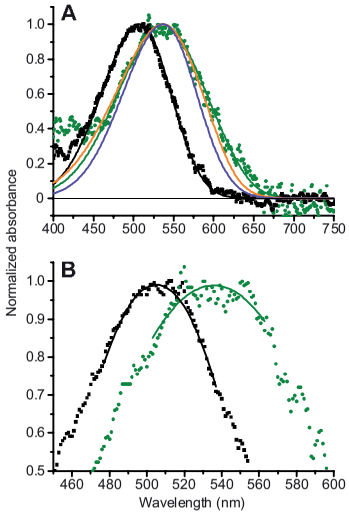


Fig. 2. Comparison of template and parabola fits as methods for estimating different chromophore ratios in rods (black) and M-cones (green). The data are averages of recordings from 28 rods and 15 M-cones in the same individual (from PYO2011). (A) Judged by template-fitting to the full spectra, rod λ_{max}=508 nm and cone λ_{max}=543.4 nm. The black curve is the template for a pure (100%) A1 pigment with λ_{max}=508 nm; the green curve is the template for 30% A1 with λ_{max}(A1)=522 nm and 70% A2 with λ_{max}(A2)=559 nm. The two dashed curves are templates for pure A1 (violet) and pure A2 (orange), both with λ_{max}=543.4 nm. (B) Judged by parabola-fitting to the domain around peak, rod λ_{max}=506 nm and cone λ_{max}=547 nm, indicating that rods have 81–92% A1 and cones have 0% A1 (i.e. pure A2) according to the assumptions explained in the Materials and methods.

Method 3: principal component analysis of mixed pigment spectra

Splitting the total variance into a number of principal components (1st, 2nd, 3rd, etc.) is a standard method in chemistry and bioinformatics. With PCA it is possible, for example, to separate spectra of different molecules present in a population of samples (e.g. Ward et al., 2003; Mäkelä et al., 2011). In work related to visual science, this approach has been used, for example, for analysis of natural illumination spectra (Judd et al., 1964) and plumage reflectance of birds (Cuthill et al., 1999). Interpretation of PCA results is not easy, however, as variation components identified purely statistically do not necessarily bear any close relation to real-world components. We still wanted to include the PCA analysis, as we think it does offer additional insight (see Table 1 and Discussion) and holds promise as a novel technique in the context of visual pigments.

We performed PCA at the population level, including in each analysis all single-cell rod or M-cone spectra recorded in the population in question. The spectra analyzed comprised 441 data points (HEL2011, RYT2011 and PYO2011), 405 data points (MAS) or 373 data points (all other 2008 spectra). The analyses included between 57 and 172 single-cell spectra for both cell types, depending on the population. Pooling all cells from a population in one analysis will of course conceal variation between individuals, but we did not have enough data to run the analysis at the level of individuals. The components were plotted and primary component graphs with clear peaks were recognized. The exact wavelengths of component peaks were determined by least-square fitting of second-order polynomials to points ±50 nm around a provisional peak estimate.

Light measurement

The spectral distribution of downwelling light (quanta $m^{-2} s^{-1} nm^{-1}$ between 400 and 750 nm) was measured with a QSM 2500 submersible quantum spectrometer (Techtum, Umeå, Sweden) (see Lindström, 2000). Measurements were taken in full daylight in September 2011 between 11:00 and 15:00 h, from the water surface all the way to the bottom in the habitats HEL, ISO, RYT and PYO at 1–5 m intervals. The spectral properties of the four other habitats were estimated by visual comparison with these and a highly red-shifted lake (Tuusulanjärvi) measured by Jokela-Määttä et al. (Jokela-Määttä et al., 2007). At least two scans were taken at each depth. One scan of the full wavelength range took approximately 1 min.

RESULTS

DNA sequencing: no amino acid difference in the rod opsin between populations

The rod opsin gene was sequenced in three specimens of each population and translated into the corresponding amino acid sequence to look for changes in the protein part of the visual pigment. A BLAST search indicated that the 856-bp DNA sequences obtained showed the highest DNA and amino acid homologies (97% in each) with the rhodopsin gene from the three-spined stickleback (*Gasterosteus aculeatus*). No DNA sequence differences were found among the 24 individuals studied, with the exception of two individuals (one from HEL and the other from MAS), which had one heterozygous synonymous change (GenBank accession no. JQ619638). Protein properties derived from sequencing of genomic DNA may in principle be subject to uncertainty because of potential differences in the transcribed products, but such variation has not been described for visual opsins. If opsins are identical, all spectral variation in the rod visual pigment must be due to varying proportions of chromophores A1 and A2. Consistent with this, the range of spectral variation in rods approximately coincided with the λ_{max} range defined by the DLH relationship for pure A1 and pure A2 chromophore in the same opsin (see Materials and methods).

MSP: four spectral classes of cones and cone opsins

In six of the eight populations studied, at least three spectral classes of cones (S, M and L) were found in addition to rods (Fig. 3). The exceptions are WHI and MAS, where no L-cones were recorded. Although a true loss shared by these two populations, which have been separated for less than a century (see Materials and methods), cannot be ruled out, it is likely that the apparent lack of L-cones is a bleaching artefact. These two populations are A2-dominated (Table 1), and the A2 version of the L-cone pigment absorbs strongly in the transmission band (>650 nm) of the edge filter used during preparation in 2008 (cf. Fig. 3B). In 2011, when we used infrared light, we did not, unfortunately, have access to specimens from these two populations.

Most of the λ_{max} variation within each of the initially delimited classes S, M and L could be explained by differing proportions of chromophores A1 and A2 (see below). However, the S-cone spectra averaged within individuals formed two non-overlapping clusters, with λ_{max} ranges of 407–412 and 426–458 nm, respectively. The dispersion of λ_{max} within clusters can be partly correlated with variation in chromophore content, as the A2 versions even of S-pigments are shifted towards longer wavelengths compared with their A1 counterparts. Hárosi's (Hárosi, 1994) formula predicts that a pigment with λ_{max}(A1)=407 nm should have λ_{max}(A2)=414 nm, which is roughly consistent with the width of the short-wavelength cluster. The 32 nm width of the long-wavelength cluster is somewhat surprising, however, as only a ca. 7 nm (Hárosi, 1994) or 12 nm (Whitmore and Bowmaker, 1989) difference between the A1 and A2 versions is predicted for pigments around 430 nm. It should be observed, however, that the cluster width refers to variation between individuals across all populations (including variation due to the low quality of many individual S-cone spectra). The population extremes for S-cones in the long-wave cluster (means ± s.e.m.) were 432.1±2.6 nm (PYO2011, N=4) and 448.3±2.3 nm (MAS, N=6). The experimental range is statistically significantly larger than the A1–A2 difference predicted by Hárosi (7 nm, P<0.05) (Hárosi, 1994), but not significantly larger than that predicted by Whitmore and Bowmaker (12 nm) (Whitmore and Bowmaker, 1989).

There is little doubt that the two clusters represent two different opsins, possibly SWS1 (giving 'violet' pigments at 355–440 nm) and SWS2 (giving 'blue' pigments at 410–490 nm) (cf. Yokoyama and Yokoyama, 2000; Hofmann and Carleton, 2009). Because of the limited material, we have no clear picture of the coexistence of SWS1 and SWS2 cones at the individual level, but all three populations studied in 2011 had both types. In 2008, recordings were obtained only from the longer-wavelength type of S-cone.

In L-cones, the population means of λ_{max} ranged from 550 nm (PYO2011) to 606 nm (HEL2011). The DLH prediction for λ_{max}(A1)=550 nm is λ_{max}(A2)=607 nm, and thus the extremes of the population means are consistent with nearly pure A1 and A2 in a

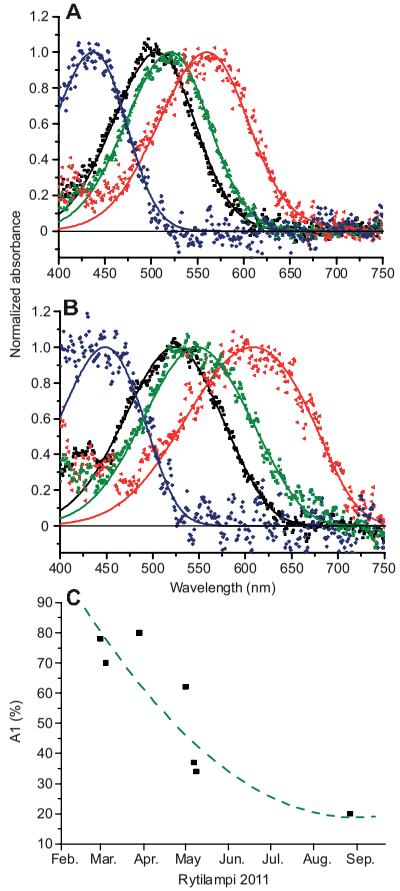


Fig. 3. (A,B) Spectra of four photoreceptor types as recorded in two individuals: one where the rods had nearly pure A1 pigment (A), and one with strongly A2-dominated rods (B). Both individuals are from the RYT population, recorded in March 2008 (A) and in May 2011 (B). All spectra have been normalized to peak absorbance 1. The curves are mixed A1–A2 templates of Govardovskii et al. (Govardovskii et al., 2000), giving the following λ_{max} values: (A) rods (black, λ_{max}=506 nm; number of cells averaged n=31), S-cones (blue, λ_{max}=437 nm, n=4), M-cones (green, λ_{max}=521 nm, n=24) and L-cones (red, λ_{max}=560 nm, n=20); (B) rods (black, λ_{max}=524 nm, n=23), S-cones (blue, λ_{max}=448 nm, n=8), M-cones (green, λ_{max}=545 nm, n=9) and L-cones (red, λ_{max}=608 nm, n=20). (C) Change in chromophore ratio of rods in the RYT population in 2011. The ordinate shows A1 percentages estimated by template-fitting to individual mean rod spectra in March (N=2), April (N=1), May (N=3) and September (N=2). The curve is a least-square parabola fitted to the points. Two individuals studied in September gave exactly the same value (20%) and therefore appear as a single point in the figure.

THE JOURNAL OF EXPERIMENTAL BIOLOGY

single opsin. For M-cones, the range of λ_{max} population means was 519–544 nm, well encompassed by the DLH relationship and thus consistent with a single opsin. However, the 25 nm range is so narrow that only one (or neither) of the boundaries can represent a pure-chromophore version of the pigment (see Materials and methods and below).

In conclusion, our MSP data suggest that the nine-spined stickleback has four classes of cone opsins expressed in four classes of cone outer segments. The L-cone outer segments recorded were most often members of M–L double cones. There were no indications that the complements of cone opsins differ between the eight populations investigated. Spectral variation within each cone class was consistent with varying chromophore proportions in a single opsin (with a cautionary reservation for the longer-wavelength S-cone).

Varying chromophore proportions in rods and cones

The populations fell into two distinct groups (hereafter referred to as groups 1 and 2) with respect to mean rod λ_{max}. In group 1, comprising populations with A1-dominated rods (Table 1, data sets 1–5), population means of rod λ_{max} were in the range of 505–511 nm. In group 2, comprising populations with A2-dominated rods (Table 1, data sets 6–10), population means of rod λ_{max} were in the range of 527–529 nm. Fig. 3 shows representative spectra of rods and cones from an A1-dominated individual (panel A) and an A2-dominated individual (panel B). High A2 in rods correlated with high A2 in cones: all spectra shown in Fig. 3B are shifted towards longer wavelengths compared with those shown in Fig. 3A.

Our procedures were designed to minimize possible variation due to seasonal and/or developmental regulation of chromophore proportions. We studied only fully developed fish that had been kept for at least 6 months in aquaria at constant temperature (15°C) under a standardized light regime (see Materials and methods). Recordings were limited to January–May for the six main populations (WHI and MAS being exceptions). Under these conditions, A1(%) was generally a sufficiently stable characteristic to allow meaningful comparisons between populations.

In only one data set (RYT2011) was a clear seasonal change between January and May observed (Fig. 3C). In fact, the data shown in Fig. 3A,B are both from RYT individuals, but recorded in different seasons: the spectra in panel A (rod λ_{max}=506 nm) were recorded in March 2008, those in panel B (rod λ_{max}=524 nm) in May 2011. In RYT2011, rod λ_{max} drifted from 510 nm in mid-March (n=2), through ca. 523.5 nm in mid-May (n=3) to 527 nm in extra recordings made in early September (n=2) especially to study this question. On the one hand, this proves that the nine-spined stickleback as a species does have the capacity to regulate chromophore proportions, although we do not know the factors that govern the shifts. On the other hand, and most importantly, none of the other data sets in our material showed a systematic shift of λ_{max} in the time window of our recordings (supplementary material Fig. S1). The properties of populations and the contrasts between them were also constant enough across the years 2008 and 2011 to support meaningful comparisons between populations (Table 1). This does not, of course, mean that there could not be chromophore changes in some or all of these populations at other times or under other conditions.

From Fig. 3A,B as well as similar sets of spectra recorded from individuals of the other populations (supplementary material Fig. S2) it is finally evident that S- and L-cone spectra tended to be of lower quality than rod and M-cone spectra because of noisier recordings and/or smaller numbers of cells recorded. The main rod/cone comparisons were therefore based on M-cones.

Quantitative estimation of chromophore proportions in rods and M-cones

Fig. 4 plots λ_{max} and A1(%) for rods and M-cones as obtained by the two independent methods of analysis, with the results of method 1 on the abscissa and those of method 2 on the ordinate. Fig. 4A,B shows the λ_{max} values for each individual fish from which a sufficient number of single-cell spectra were obtained, different populations being distinguished by different symbols. There is fair agreement between values obtained by the two methods (method 1, r^2=0.966 for rods and 0.933 for M-cones; method 2, r^2=1.008 for rods and 0.951 for M-cones). In the rod data (panel A), the segregation into a short-wavelength group 1 and a long-wavelength group 2 is immediately evident, whereas the distribution of M-cone λ_{max} is less clearly bimodal.

Fig. 4C,D correspondingly relates population-level estimates of A1(%) according to methods 1 and 2. In the rod data, the segregation of group 1 (A1-dominated, upper right cluster) and group 2 (A2-dominated, lower left cluster) is again obvious, except for one outlier (WHI, green star in Fig. 4C). This deviant data point reflects the fact that method 1, when applied to very noisy data (poorly defined spectral shape), is not good at disentangling the two fitting parameters λ_{max}(A1) and A1(%) (see Materials and methods). Thus, for WHI rods the (unconstrained) best fit was achieved with mean λ_{max}(A1)=520 nm, giving A1(%)≈80%. Fitting the same data under the constraint λ_{max}(A1)=504.8 nm (the lowest rod population mean) gives mean A1(%)=22%; however, several of the individual spectra are then poorly fitted even by the pure A2 template (0% A1). Although the latter strategy might have been a reasonable alternative when fitting rod spectra, as the opsin was known to be the same in all populations, we chose not to do so in order to preserve the independence of methods 1 and 2 and to analyze rods and M-cones in the same way. It is worth noting that this problem, the trade-off of λ_{max} and A1:A2 in fitting, is encountered whenever 'mixed' spectra are analyzed without prior knowledge of the components, for example, when studying a new species.

As previously pointed out, method 2 is not susceptible to this type of error. Instead, it requires a prior assumption regarding the λ_{max} of the pure components, and is sensitive to the accuracy of that assumption (to which method 1 is immune). In Fig. 4C,D, 45 deg lines that would correspond to perfect correlation of values obtained by the two methods have been drawn for visual guidance; the obvious deviations are indicative of their different error sources. Yet the correlation is good enough to make it meaningful to average them to yield a single final estimate for A1(%) in each data set (Table 1, Fig. 5).

Fig. 5 summarizes the A1(%) of rods and M-cones for each of the data sets. The estimates from methods 1 and 2 were averaged for each individual, and the bars in the histogram give means ± s.d. of (arcsine-transformed) individual estimates. The rod–cone differences within data sets were tested by two-way ANOVA (photoreceptor type; data set) on the arcsine-transformed values. The rod–cone differences in chromophore proportions were statistically significant for all data sets in group 1 and for none of those in group 2 (at the P<0.05 level, i.e. satisfying the criterion P<0.0045 prior to Bonferroni correction for n=11).

Method 3: decomposing mixed spectra by principal component analysis

For PCA, all single-cell spectra (either from rods or from M-cones) were pooled within each population. It should be realized that PCA gives variance fractions explained by spectral components that are identified on purely statistical grounds, not measures of

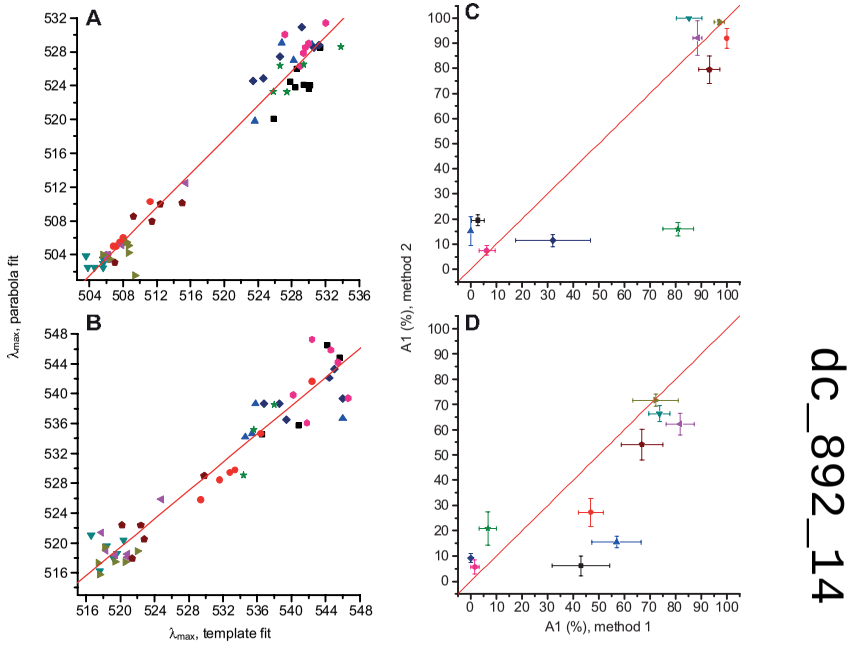


Fig. 4. (A,B). Estimates of λ_{max} of rods (A) and cones (B) obtained by fitting Govardovskii et al. (Govardovskii et al., 2000) templates (abscissa) and parabolas (ordinates) to spectra of individual fish. Each symbol type indicates one data set as follows (enumerated in the same order as in Table 1): Group 1: ABB2008 (green triangle pointing to the right), ISO2008 (brown pentagon), PYO2008 (turquoise inverted triangle), PYO2011 (red circle), RYT2008 (pink triangle pointing to the left); Group 2: BOL2008 (blue diamond), HEL2008 (blue upright triangle), HEL2011 (black square), WHI2008 (green star), MAS2008 (pink hexagon). The equations of the least-square lines fitted to the data are: (A) rods, y=1.008x+6.324 (r^2=0.966); (B) cones, y=0.951x+24.964 (r^2=0.934). (C,D) Estimates of A1(%) of rods (C) and cones (D) obtained by method 1 (abscissa) and method 2 (ordinates); data are means ± s.d. for each data set. Symbols for the data sets as in A and B. The lines are 45 deg lines drawn for visual guidance. See Materials and methods and Results for details.

chromophore proportions. Thus PCA components need not correspond to real visual-pigment spectra. Yet if there is little variation in chromophore proportions between cells, the primary PCA component is usually close to the main peak (α-band) of the recorded spectrum (capturing variation in absolute amplitude between recorded spectra). In contrast, if the chromophore proportions vary between cells, this is liable to emerge as a PCA component resembling the A1–A2 difference spectrum. Because there are many factors causing variation in recorded spectra, however, even the variance of recordings from a single homogeneous visual pigment (with a single chromophore) may emerge as consisting of several rather similar PCA components. It was common in our analysis to obtain two visual-pigment-like PCA components that differed only in the β-band domain, although the peak was in the α-band. To make Table 1 more readable, we excluded components explaining a lower percentage of the variance if there was another component within 10 nm that explained a higher percentage. With this qualification, Table 1 shows the peak wavelengths of all major PCA components ('major' defined as explaining at least 1% of the total variance), and the percent of the total variance explained by each.

For ABB, BOL and MAS (data sets 1, 6 and 10), PCA analysis had little to add, as the only major component found in rods as well as cones was similar to the full recorded spectrum. Several of the others, however, merit comment. The A1–A2 difference spectra of Govardovskii et al. (Govardovskii et al., 2000) templates for λ_{max}(A1)=504.8 nm (rods) and λ_{max}(A1)=512.5 nm (M-cones) have broad peaks at 530–610 nm (maximum at 568 nm) and 540–630 nm (maximum 579 nm), respectively. In the ISO and the RYT2011 data sets (nos 2 and 11), the second principal components in rods and cones are consistent with these. Intriguingly, RYT2008 (data set 5) also has a rod component suggestive of the A1–A2 difference spectrum. This may signal an incipient rod chromophore switch in the RYT population also in the year 2008 (like in 2011), detected by PCA, although still too small to be detected as a λ_{max} shift or a significant change in spectral shape.

The components identified by PCA in this data set are illustrated in Fig. 6. It is also the only case where PCA found a greater number

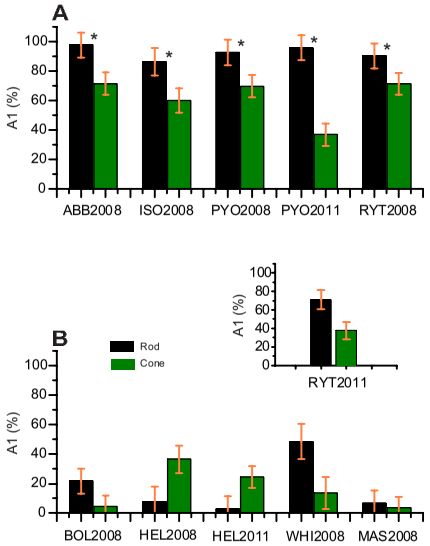


Fig. 5. A1 percentages of rods (black bars) and M-cones (green bars) in populations of group 1 (A), group 2 (B), and the data set RYT2011 reflecting a non-stationary situation (inset). Stars indicate rod–cone differences that are statistically significant at the P<0.05 level (observing the Bonferroni correction for non-independent testing of 11 data sets: P=0.05/11=0.0045). In group 1, all differences were significant (P<0.0012). In group 2, only WHI2008 was marginally significant (P=0.0054); for all others, P>0.015. Error bars are ±s.d. for the arcsine-transformed data.

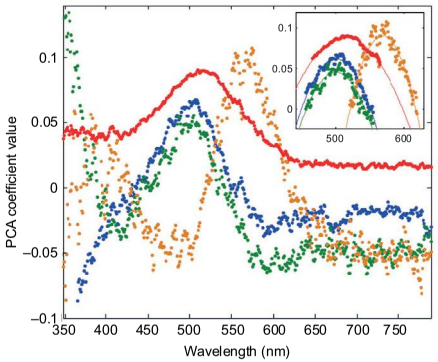


Fig. 6. Determination and interpretation of the principal variance components of a mixed spectrum. The full spectrum was that of rods in the data set RYT2008 (no. 5 in Table 1), peaking at ca. 507 nm. The first variance component (red curve, explaining 66.8% of the variance) is roughly congruent with the full spectrum. The next two components both peaking near 501 nm (blue and green curves, explaining 13.5 and 5.4%, respectively) are consistent with the pure A1 rod pigment. The fourth variance component, peaking at ca. 561 nm (yellow curve, explaining 2.1%), may be attributed to the A1–A2 difference spectrum. All other components explained less than 1% of the total variance and were therefore neglected (see Materials and methods and Results for details). Components 1, 2 and 4 have been inverted for the figure. The inset shows how the peak wavelengths of the component spectra were determined by least-square fitting of parabolas over the top.

of major variance components in rods than in cones (the cone PCA in the same data set gives the full pigment spectrum as the only major component).

The results for data set PYO2011 (no. 4 in Table 1) are also interesting, as M-cones yielded no less than four major PCA components. The first one (peaking at 532.5 nm) simply represents the full spectrum. The two following ones (519.8 and 546.8 nm) resemble the spectra of the pure chromophore components, and the last one (589.5 nm) is consistent with the M-cone A1–A2 difference spectrum. Rod spectra yieled two PCA components, which may be associated with the full spectrum and the rod A1–A2 difference spectrum, respectively (507.2 and 574.2 nm).

Chromophore proportions may be different in L- and M-cones

Our recordings from other cone types are not extensive enough to support comprehensive comparisons of chromophore proportions across populations. About the two classes of S-cones we can only say that their A1:A2 ratios varied, quite possibly over the full range from 0% to 100% A1 (see above). The L-cone data, however, do allow some definite conclusions, despite the fact that we have to limit ourselves to the 2011 recordings, as only they can be regarded as spectrally unbiased. They comprised one population from group 2 (HEL2011) and two from group 1 (PYO2011 plus the labile RYT2011). We based the analysis of chromophore proportions in L-cones on method 2, as the observed population extremes of λ_{max} were consistent with those expected for an A1–A2 pair [λ_{max}(A1)≈550 nm and λ_{max}(A2)≈607 nm, see above].

The results from PYO2011, the only stable group 1 data set among these, are the most interesting. Mean λ_{max} in L-cones was 550.3±1.9 nm (N=7, n=133). This corresponds to 100% A1, congruent with the rods, whereas the M-cones of the same individuals were estimated to have only 40% A1 (see Fig. 5A, Table 1). The L–M difference in A1(%) is statistically significant (two-tailed P<0.001 on a t-test for paired, arcsine-transformed values).

The group 2 data set HEL2011 conforms to the notion that A2 dominance in rods correlates with A2 dominance in all cone types (cf. Fig. 3). Population mean λ_{max} of L-cones was 606.4±3.7 nm (N=5, n=20), indicating practically 0% A1 (compare with an estimated 3% in rods and 27% in M-cones of the same data set). Fig. 7 shows the population-mean spectra of M-cones and L-cones of PYO2011 (panel A) and HEL2011 (panel B).

As previously mentioned, RYT2011 was in a state of transition. Individual mean values of L-cone λ_{max} went from ca. 548 nm in March (N=2, n=12) to ca. 602 nm in May (N=2, n=21). The corresponding A1 percentages would be 100 and 9%, respectively, suggesting a switch even more complete than in the rods (which went from 76 to 32% A1; see Fig. 3C).

DISCUSSION

Differences in rod absorbance spectra are due to variable chromophore ratios

DNA sequencing revealed no amino acid variation in the rod opsin gene. Thus variation in λ_{max} of the rod visual pigment must be due

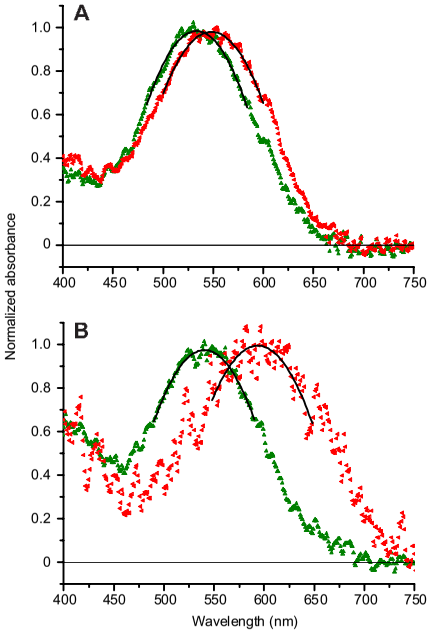


Fig. 7. Comparison of M-cone (green) and L-cone (red) spectra averaged across all individuals in two of the data sets. (A) Group 1 population PYO (no. 4 in Table 1), with estimated A1(%)≈40% for M-cones and 100% for L-cones (both based on fits to spectra of the same seven individuals). The spectra have been fitted with parabolas ±50 nm around a provisional peak; these give λ_{max}≈533 nm (M) and 549 nm (L). (B) Group 2 population HEL (no. 8 in Table 1), with estimated A1(%)≈23% for M-cones and 0% for L-cones (the latter based on fits to spectra of five individuals). The parabola fits shown indicate λ_{max}≈541 nm (M-cones) and 596 nm (L-cones). Note that these λ_{max} values estimated from the population-level average spectra deviate slightly from those calculated as means of the λ_{max} values obtained by separate fitting of each individual spectrum in the same data set (i.e. the values given in the Discussion and Table 1).

to varying proportions of chromophores A1 and A2. Consistent with this, the observed variation range of rod λ_{max} closely matched the DLH prediction for a visual pigment that has a λ_{max} of ~504 nm when coupled to the A1 chromophore.

The populations fell into two distinct groups differing with respect to λ_{max} and the dominant chromophore of the rod pigment: short-wavelength-sensitive, A1-dominated (group 1) and long-wavelength-sensitive, A2-dominated (group 2; see Table 1, Fig. 5). Reinvestigation of three populations (PYO, RYT from group 1 and HEL from group 2) after a 3 year interval indicated that the differences were quite robust over the years for fish that had been kept under the same standardized conditions and studied at approximately the same time of the year. The differences invite a simple generalization based on habitat type. Group 1 consists of populations from freshwater habitats cut off from the sea thousands of years ago (Donner, 1995; Eronen et al., 2001). Group 2 consists of salt- or brackish-water populations plus a population from Mashinnoye pond, which has been separated from the sea for a few decades only (see Materials and methods). This apparent sea/lake dichotomy was unexpected and may be due to chance, as it resulted from a sample of only eight populations.

At least three points should be noted when judging this result. First, as indicated by RYT2011 (Fig. 3C), the nine-spined stickleback (like many other fishes) possesses the capacity to regulate the chromophore content of visual pigments on a physiological time scale, although the factors that govern the balance in this species are unknown. The spectral 'snapshots' considered here certainly do not give a full picture of how chromophore proportions in freshwater and brackish-water populations may change under natural conditions. Second, the fish from all populations were kept in freshwater aquaria under similar conditions for several months before experiments, and the same saline was used for all retinas during preparation and recording. Thus there cannot have been differential effects of ions directly on the visual pigment. Third, the common generalization that sea and lake fishes differ with respect to chromophore usage goes in the opposite direction (our sea populations have more A2 chromophore and the lake populations have more A1 chromophore, which is against the general trend) (Wald, 1937; Wald, 1939; Bridges, 1972; Jokela-Määttä et al., 2007; but see Wald, 1941; Schwanzara, 1967), which generally makes more sense, as spectral transmission in lakes tends to be red-shifted (which would favour A2) compared with that in seas (Jerlov, 1976).

Even though they cannot at present be given a clear ecological interpretation, the differences between groups 1 and 2 indicate genetic differences (with the possible exceptions of WHI and MAS, which were studied in a different season than the other populations). These may have to do with norms of reaction to some environmental factors, intrinsic seasonal rhythms or the degree of plasticity in chromophore proportions. We do not know, but we can make theoretical predictions about the relative performance of A1 and A2 versions of the pigments in their present light environments (see below).

Four spectral classes of cones

We found four spectral classes of cones, each consistent with one of the four main classes of cone opsin genes in the vertebrate lineage (*SWS1*, *SWS2*, *RH2* and *M/LWS*) (cf. Yokoyama and Yokoyama, 2000; Hofmann and Carleton, 2009). Our failure to find one or the other of the cone classes in some populations was most probably due to bleaching (in L-cones) or mere chance (for S-cones), and cannot be taken to suggest that populations differ in their cone complements.

The ranges of λ_{max} variation within each of the four cone classes, as well as the shapes of spectra as analyzed in M-cones, were consistent with the idea that the only tuning mechanism was the varying proportions of chromophores A1 and A2. Thus we analyzed the cone spectra, like the rod spectra, on the assumption that the opsins did not differ between populations.

Different chromophore proportions in rods, M-cones and L-cones

The most interesting discovery was that all populations in group 1 had a significantly higher fraction of A2 chromophore in the M-cones than in the rods. Mechanistically, this might be implemented by the recently described delivery of 11-*cis* chromophore to cones from Müller cells (Wang et al., 2009; Wang and Kefalov, 2011). This makes cones partly independent of the retinal pigment

doi:10.1242/jeb.069674

epithelium, previously thought to be the only source for all photoreceptors (Fain et al., 1996; cf. Reuter et al., 1971). Functionally, the privileged supply of 11-*cis* chromophore to cones has been interpreted as a means of ensuring fast and reliable regeneration of cone pigment without competition with rods (e.g. Suzuki et al., 1985). In the present study, it also emerges as a potential way of achieving independent spectral tuning of rod and cone visual pigments. For this to work, one must assume independently regulated A1 to A2 conversion (by a regulated retinol 3,4-dehydrogenase) somewhere *en route* to the visual pigment in the cone outer-segment membranes. Chromophore selectivity could also be due to cone-specific mechanisms (cone-specific localization of the dehydrogenase, or 11-*cis* transport or uptake). Any mechanistic scheme must remain speculative at this stage, and would also have to accommodate the fact that L-cones can differ from M-cones in chromophore content. Selective chromophore handling by different types of rods is known from several species of deep-sea fish (Bowmaker et al., 1988; Crescitelli, 1989).

Our conclusions are based on A1:A2 estimation by two independent methods, which gave essentially concurrent results (Fig. 4), whereas their potential error sources were mainly different (cf. Materials and methods). The only assumption common to both that could potentially introduce correlated bias is the coupling of λ_{max}(A1) and λ_{max}(A2) of pigment pairs by the Hárosi (Hárosi, 1994) relationship. Alternative and slightly different coupling relationships have been proposed previously (Whitmore and Bowmaker, 1989; Parry and Bowmaker, 2000). All of these are purely phenomenological descriptions of certain data sets, of which the set used by Hárosi (Hárosi, 1994) is the most extensive. Moreover, in the wavelength domain critical for stickleback rods and M-cones [λ_{max}(A1) between 500 and 520 nm], the Hárosi predictions for λ_{max}(A2) are rather precisely intermediate between those of the two others. The maximal difference in predicted λ_{max}(A2) in this domain is ±5 nm, which would slightly change our A1:A2 estimates, but by too little to be of any significance for the conclusions. Around L-cone λ_{max}(A1) (≈550 nm), the Hárosi and Whitmore–Bowmaker relationships give practically identical predictions, whereas λ_{max}(A2) according to the Parry–Bowmaker relationship is lower by more than 5 nm in this domain. The difference would not significantly affect the comparison of A1(%) in M- and L-cones, however.

The performance of A1 and A2 rods and cones in the light environments of the sticklebacks

Rods

'Optimal' performance of a visual pigment depends on what is assumed to be its main task. For rod pigments, the benchmark often adopted is absolute visual sensitivity. This requires maximizing the SNR in very dim light, where the dominant noise is 'dark noise', intrinsic to the visual neurons, rather than noise arising from the Poisson fluctuations of the photon flux (Barlow, 1956). In rods of many species, the main intrinsic noise component liable to interfere with light detection comes from randomly occurring spontaneous (thermal) activations of visual-pigment molecules (Baylor et al., 1980; Aho et al., 1988; Ala-Laurila et al., 2004a; Luo et al., 2011). Maximizing the SNR then requires not only that the visual pigment catch photons efficiently, but also that it be thermally stable. Because the rate of thermal activation of visual pigments correlates strongly with red sensitivity (Luo et al., 2011), optimization of a pigment for light environments rich in long wavelengths faces an intricate trade-off. The advantage of a red-shift that increases quantum catch (QC) in a certain environment may be offset or even reversed in terms of SNR by the associated increase in thermal noise. Increasing noise is an inevitable correlate when red-tuning is achieved by an A1 to A2 chromophore switch in the same opsin (Donner et al., 1990; Ala-Laurila et al., 2004b; Ala-Laurila et al., 2007), whereas red-tuning by opsin changes may combine spectrally relevant amino acid substitutions with other (thermally relevant) amino acid substitutions that mitigate the decrease in thermal stability (Fyhrquist et al., 1998; Koskelainen et al., 2000).

However, the main goal for rod vision need not always be to maximize absolute sensitivity, but to support good vision at slightly higher (still scotopic) light levels. From some mean luminance upwards, photon fluctuations will surpass thermal activation as the main source of pigment-originated noise even in photoreceptors with 'noisy' pigments. Above that level, spectral tuning for increased QC will always increase SNR. (The critical light level in itself will, of course, depend on the noisiness of the pigment.) This idea has been invoked to explain general rod/cone differences (Barlow, 1957; Lythgoe, 1984), but may also be applied to variation in rod properties.

Fig. 8A shows a family of spectra (normalized to unity at peak) describing the downwelling light at different depths in one of the habitats, HEL, measured in daylight in September 2011 (see Materials and methods). The spectra (peaking around 570 nm) are quite representative of all except one (ABB) of the habitats in the present study. Other spectra measured at the same time were ISO (peak transmission at ca. 575 nm), RYT (565 nm) and PYO (565 nm). The four remaining habitats were not measured, but BOL, WHI and MAS appeared similar to the others as judged by eye. The only obvious outlier is ABB, a humic pond with strongly red-shifted transmittance.

The vertical lines in Fig. 8 mark λ_{max} of the pure A1 (violet) and pure A2 (orange) versions of the rod pigment. Two points are worth noting. First, regardless of chromophore, rod absorbance spectra are positioned at far too short wavelengths to make efficient use of the available light under most illuminations (except maybe under the blue skylight at dusk or dawn), a well-known situation in coastal and freshwater fish species (Lythgoe, 1979; Lythgoe, 1984). Second, 100% A2 chromophore would provide the best spectral match of the rod pigment to the downwelling light in all the water bodies of the present study. Why, then, do not all eight populations use A2 in rods? The most reasonable explanation is the noisiness of A2 pigments.

Jokela-Määttä et al. (Jokela-Määttä et al., 2007) have calculated relative QC (QC_{rel}) and conceptual SNR at the absolute threshold (SNR_{dark}) of A1 and A2 visual pigments as functions of λ_{max} for five different aquatic environments, some of which are very similar to the ones used in the present study, and summarized the results in graphic form (their fig. 3). Calculation of QC is straightforward (convolution of pigment absorptance spectra with illumination spectra). For calculation of SNR_{dark} it may be assumed that the frequency of thermal pigment activations is a monotonically increasing function of λ_{max}, $F(\lambda_{max})$, as modelled by Ala-Laurila et al. (Ala-Laurila et al., 2004a) and essentially confirmed by Luo et al. (Luo et al., 2011). The noise intrinsic to the visual pigment is proportional to the standard deviation of Poisson-distributed thermal 'dark' events within some integration time t_i. Because the Poisson standard deviation is equal to the square root of the mean (relative), SNR_{dark} may be defined as:

$$SNR_{dark} = QC_{rel} / \sqrt{[F(\lambda_{max}) \times t_i]} . \quad (1)$$

As only relative values interest us here, the integration time t_i may be used as an arbitrary proportionality constant. SNR_{dark} is a relevant measure of pigment performance at the very lowest light levels, where thermal activations dominate over photon fluctuations as a source of pigment-originated noise. Estimates of QC_{rel} and SNR_{dark} for the A1 and A2 versions of stickleback rod pigment in the present habitats (excepting ABB) can be interpolated from two of the curve sets in Jokela-Määttä et al. (Jokela-Määttä et al., 2007), which refer to light spectra peaking at 560 nm (their *B*) and 580 nm (their B_b), respectively. The estimates suggest that rods with the A2 chromophore would catch ca. 30% more quanta than those with the A1 chromophore on average in light environments like HEL (Fig. 8A). Judged by SNR_{dark}, the relationship is reversed: at the absolute threshold, A1 rods would perform more than 50% better than A2 rods on average. An ultra-adaptationist hypothesis might be that group 2 sticklebacks 'want to' see well at moderate scotopic light levels (and thus use A2), whereas group 1 fish 'want to' maximize absolute sensitivity (and thus use A1). (In humic lakes such as ABB, however, A2 would be superior in terms of both QC_{rel} and SNR_{dark}.) To evaluate this hypothesis, one would have to study the behaviour of the fish in their natural habitats in different seasons.

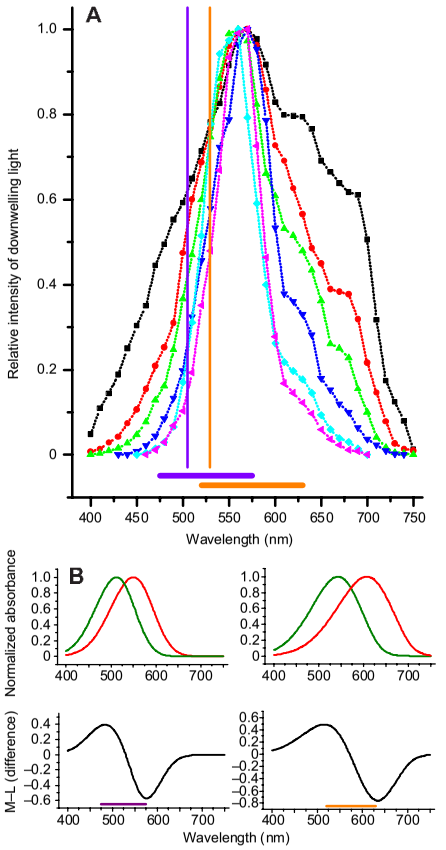


Fig. 8. Visual pigments with A2 chromophore provide better matches to the present light environments of the sticklebacks than those with A1 chromophore. The spectral distribution of downwelling light at different depths in the habitat HEL (representative of all the habitats except the red-shifted ABB) is here compared with rod λ_{max} (vertical lines: violet, A1; orange, A2), and domains of good wavelength discrimination as derived from by the M–L cone difference spectrum (horizontal bars: violet, A1; orange, A2). (A) The curve family gives spectra of downwelling light (quanta per unit area and unit time, relative units) measured in daytime in September 2011 at depths of 1 (broadest spectrum), 2, 3, 5, 8 and 9.4 m (narrowest spectrum). (B) How the domains of good wavelength discrimination are derived from M–L difference spectra. Top row: spectra of M-cones (green) and L-cones (red) with A1 (left) and A2 (right) chromophore. Bottom row: M–L difference spectra (black curves). The steep parts that correspond to domains of high wavelength resolution span ca. 475–575 nm for A1 (violet bar) and ca. 520–630 nm for A2 (orange bar).

Cones

In brighter light, the disadvantages brought by the stronger 'dark noise' and lower photosensitivity of A2 compared with A1 pigments (cf. Dartnall, 1972; Bridges, 1972) are expected to matter much less. Hence, cones may have less reason than rods to avoid the A2 chromophore, and optimal chromophore proportions in a given light environment may be quite different for cones and rods. If the task is achromatic contrast discrimination by a single type of cone, QC is the relevant measure of pigment performance in brighter light. Estimates based on the modelling of Jokela-Määttä et al. (Jokela-Määttä et al., 2007) (see above) suggest that the A2 version of the M-cone pigment achieves 30% higher QC than its A1 counterpart in a light environment such as HEL (Fig. 8A). For the L-cone pigment, however, the A1 version remains superior, as QC_{A1}/QC_{A2}=1.5. Yet the L-cones of the local HEL population had nearly 100% A2 (see Results).

This apparent paradox is resolved, however, when the performance of L-cones is related to their main task, colour vision, where the elementary operation is wavelength discrimination by comparison of signals from M- and L-cones. Fig. 8B illustrates how a measure of wavelength discrimination based on subtractive coupling can be derived from M–L difference spectra. Discrimination is best in the domains where the M–L difference signal changes steeply as function of wavelength [i.e. the derivative $|d(M–L)/d\lambda|$ is large]. The main domains are marked by a violet bar for the A1 pigments and by an orange bar for the A2 pigments (these bars have been reproduced in panel A). On the whole, the M–L pair using A2 pigments is seen to match the light spectra much better than that using A1. A crucial factor is the protracted long-wavelength limb of the M-cone A2 pigment, which extends the upper bound of the domain of good discrimination from 575 to 630 nm. However, it must be remembered that the A1 to A2 switch will also red-shift the short-wave limb of the M-cone, which could in turn compromise wavelength discrimination at the interface with S-cones. A full analysis of M-cone performance in wavelength discrimination would have to observe the demands of both 'red–green' and 'yellow–blue' opponency, and a full functional description would have to be formulated in terms of the resultant colour space (e.g. Vorobyev and Osorio, 1998).

Here, however, we shall consider only one section through the complex discrimination space. Assume that A2 in M-cones is fixed at 100%. How does M–L discrimination change when the percentage of A2 in L-cones [denoted L(A2)] rises from 0 to 100%? In Fig. 9, the inset shows a family of M–L difference spectra at 10% intervals of L(A2). Besides the width of the discrimination domain, the crucial variable is the steepness of the difference spectrum within that domain [$|d(M–L)/d\lambda|$; see above], which defines resolving power. The steepness there is nearly constant and can be described by straight

lines. The slope coefficients β of these lines (suitably normalized) may be used as relative measures of wavelength discrimination. These have been plotted as functions of L(A2) in Fig. 9 (violet curve in the main panel), showing that wavelength resolution improves monotonically with increasing L(A2). This function would hold at high illumination levels, where photon fluctuations dominate over thermal pigment activations as a source of pigment-originated noise. At low light levels, however, the intrinsic noisiness of A2 pigments in general and L(A2) pigment in particular cannot be neglected, and in the limiting case, the thermal pigment activations are completely dominant compared with photoactivations. The thermal activation rate of the L-cone A2 pigment may be assumed to be some 50 times higher than that of either M-cone A2 or L-cone A1 pigments (cf. Ala-Laurila et al., 2004a; Ala-Laurila et al., 2007; Luo et al., 2011). Therefore, almost all (>90%) of the thermal activations liable to blur the M–L difference signal will be due to L(A2) pigment when its fraction of rises above some 20%, and the noisiness of the other pigments may be neglected. The (relative) wavelength discrimination function at the lowest light levels is then given by (cf. Eqn 1):

$$\mathrm{SNR}_{\mathrm{dark}} = C\beta / \sqrt{[\mathrm{L(A2)\%}]}, \qquad (2)$$

where C is an arbitrary proportionality constant. Eqn 2 is plotted as the lower curve (blue) in Fig. 9, giving wavelength discrimination as function of A2 percentage in L-cones at very low light levels. Even then, there is significant improvement with increasing L(A2) at least up to 60–70%. As the mean light intensity increases, photon fluctuations gradually cause an increase in pigment-originated noise, and the 'operating function' will gradually shift from the blue to the violet curve. The main point of this example is to show that M–L wavelength discrimination may indeed benefit from high proportions of A2 in both M- and L-cones. In light environments where high absolute (scotopic) sensitivity requires high proportions of A1 in rods, there appear to be good functional reasons to use higher proportions of A2 in cones than in rods.

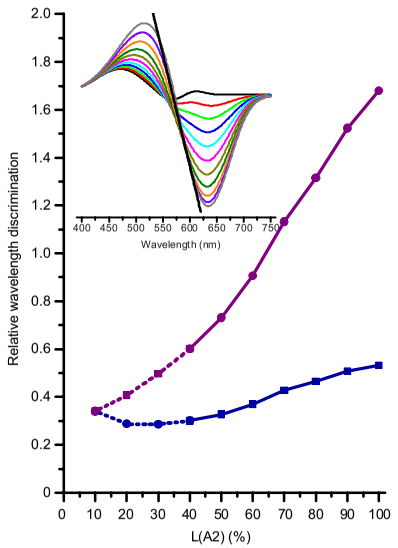


Fig. 9. Wavelength discrimination based on M–L cone difference spectra as a function of A2 percentage in the L pigment [L(A2)], when the M pigment is pure A2. The inset shows M–L difference spectra at 10% intervals of L(A2) (obtained as illustrated in Fig. 8B). The straight line exemplifies how the steepness of the slope is read from each curve. The slope coefficients are used as relative measures of wavelength resolution, plotted in the main graph (violet). However, below 40% L(A2), the discrimination domain gets narrower; this zone is marked by dashed lines. The square root of [L(A2)%/10] is taken as a relative measure of pigment 'dark' noise (see Discussion). When each slope coefficient is divided by this noise measure, a relative measure of the intrinsic SNR for wavelength discrimination is obtained [blue curve, anchored to the violet curve at 10% L(A2)]. The violet curve would describe the relationship in bright light, the blue curve at the lowest light levels.

Conclusions

Differences in visual-pigment absorptance spectra between eight populations of nine-spined stickleback were explained by differences in the proportions of chromophores A1 and A2. Within populations, chromophore proportions differed significantly between rods and M-cones, and between M-cones and L-cones. All populations with A1-dominated rods had significantly higher proportions of A2 in M-cones than in rods. Mechanistically, this indicates selective processing of 11-*cis* chromophore for different photoreceptor types. Functionally, higher A1 proportions in rods are consistent with a more compelling need to limit thermal noise, whereas higher A2 proportions in M-cones provide a better spectral match to prevailing light environments. The results suggest a possible ecological role for the different chromophore delivery pathways described recently.

ACKNOWLEDGEMENTS

We wish to thank Prof. David Hunt and Dr Wayne Davies for kindly providing the primer sequences and protocols for PCR, and Dr Magnus Lindström for help in measuring the spectra of downwelling light in the habitats. We are grateful for the skilful technical assistance of Dr Mirka Jokela-Määttä and Ms Sophie Aminoff.

FUNDING

This work was supported by the Academy of Finland [grant number 128081 to K.D. and numbers 129662 and 134728 to J.M.]; the Waldemar von Frenckell Foundation, The Societas Scientiarum Fennica and the Otto A. Malm Foundation [to J.P.]; and the University of Helsinki [to P.S.].

REFERENCES

Aho, A.-C., Donner, K., Hydén, C., Larsen, L. O. and Reuter, T. (1988). Low retinal noise in animals with low body temperature allows high visual sensitivity. *Nature* **334**, 348-350.
Ala-Laurila, P., Saarinen, P., Albert, R., Koskelainen, A. and Donner, K. (2002). Temperature effects on spectral properties of red and green rods in toad retina. *Vis. Neurosci.* **19**, 781-792.
Ala-Laurila, P., Donner, K. and Koskelainen, A. (2004a). Thermal activation and photoactivation of visual pigments. *Biophys. J.* **86**, 3653-3662.
Ala-Laurila, P., Pahlberg, J., Koskelainen, A. and Donner, K. (2004b). On the relation between the photoactivation energy and the absorbance spectrum of visual pigments. *Vision Res.* **44**, 2153-2158.
Ala-Laurila, P., Donner, K., Crouch, R. K. and Cornwall, M. C. (2007). Chromophore switch from 11-cis-dehydroretinal (A2) to 11-cis-retinal (A1) decreases dark noise in salamander red rods. *J. Physiol.* **585**, 57-74.
Archer, S., Hope, A. and Partridge, J. C. (1995). The molecular basis for the green-blue sensitivity shift in the rod visual pigments of the European eel. *Proc. R. Soc. B* **262**, 289-295.
Barlow, H. B. (1956). Retinal noise and absolute threshold. *J. Opt. Soc. Am.* **46**, 634-639.
Barlow, H. B. (1957). Purkinje shift and retinal noise. *Nature* **179**, 255-256.
Barlow, H. B. (1958). Temporal and spatial summation in human vision at different background intensities. *J. Physiol.* **141**, 337-350.
Baylor, D. A., Matthews, G. and Yau, K. W. (1980). Two components of electrical dark noise in toad retinal rod outer segments. *J. Physiol.* **309**, 591-621.
Beatty, D. D. (1975). Rhodopsin-porphyropsin changes in paired-pigment fishes. In *Vision in Fishes: New Approaches in Research* (ed. M. A. Ali), pp. 635-644. New York: Plenum Press.
Bowmaker, J. K., Dartnall, H. J. A. and Herring, P. J. (1988). Longwave-sensitive visual pigments in some deep-sea fishes: segregation of 'paired' rhodopsins and porphyropsins. *J. Comp. Physiol. A* **163**, 685-698.
Bridges, C. D. B. (1972). The rhodopsin-porphyropsin visual system. In *Handbook of Sensory Physiology VII/1: Photochemistry of Vision* (ed. H. J. A. Dartnall), pp. 417-480. Berlin: Springer-Verlag.
Browman, H. I. and Hawryshyn, C. W. (1994). The developmental trajectory of ultraviolet photosensitivity in rainbow trout is altered by thyroxine. *Vision Res.* **34**, 1397-1406.
Carleton, K. L. and Kocher, T. D. (2001). Cone opsin genes of african cichlid fishes: tuning spectral sensitivity by differential gene expression. *Mol. Biol. Evol.* **18**, 1540-1550.
Carlisle, D. B. and Denton, E. J. (1959). On the metamorphosis of the visual pigments of *Anguilla anguilla* (L.). *J. Mar. Biol. Assoc. UK* **38**, 97-102.
Crescitelli, F. (1989). The visual pigments of a deep-water malacosteid fish. *J. Mar. Biol. Assoc. UK* **69**, 43-51.
Cuthill, I. C., Bennett, A. T. D., Partridge, J. C. and Maier, E. J. (1999). Plumage reflectance and the objective assessment of avian sexual dichromatism. *Am. Nat.* **153**, 183-200.
Dartnall, H. J. A. (ed.) (1972). Photosensitivity. In *Handbook of Sensory Physiology*, Vol. VII/I, pp. 122-145. New York: Springer-Verlag.
Dartnall, H. J. A. and Lythgoe, J. N. (1965). The spectral clustering of visual pigments. *Vision Res.* **5**, 81-100.
Donner, J. (1995). *The Quaternary History of Scandinavia*. Cambridge: Cambridge University Press.
Donner, K., Firsov, M. L. and Govardovskii, V. I. (1990). The frequency of isomerization-like 'dark' events in rhodopsin and porphyropsin rods of the bull-frog retina. *J. Physiol.* **428**, 673-692.
Duan, W. and Fuerst, P. A. (2001). Isolation of a sex-linked DNA sequence in cranes. *J. Hered.* **92**, 392-397.
Eronen, M., Glückert, G., Hatakka, L., van de Plassche, O., van der Plicht, J. and Rantala, P. (2001). Rates of Holocene isostatic uplift and relative sea-level lowering of the Baltic in SW Finland based on studies of isolation contacts. *Boreas* **30**, 17-30.
Fain, G. L., Matthews, H. R. and Cornwall, M. C. (1996). Dark adaptation in vertebrate photoreceptors. *Trends Neurosci.* **19**, 502-507.
Fyhrquist, N., Donner, K., Hargrave, P. A., McDowell, J. H., Popp, M. P. and Smith, W. C. (1998). Rhodopsins from three frog and toad species: sequences and functional comparisons. *Exp. Eye Res.* **66**, 295-305.
Gonda, A., Herczeg, G. and Merilä, J. (2011). Population variation in brain size of nine-spined sticklebacks (*Pungitius pungitius*) – local adaptation or environmentally induced variation? *BMC Evol. Biol.* **11**, 75.
Govardovskii, V. I. and Zueva, L. V. (1988). A simple highly sensitive recording microspectrophotometer. *Tsitologiia* **30**, 499-502.
Govardovskii, V. I. and Zueva, L. V. (2000). Fast microspectrophotometer for studying the photolysis of visual pigments *in situ*. *Sensornye Sistemy* **14**, 288-296.
Govardovskii, V. I., Fyhrquist, N., Reuter, T., Kuzmin, D. G. and Donner, K. (2000). In search of the visual pigment template. *Vis. Neurosci.* **17**, 509-528.
Hargrave, P. A., McDowell, J. H., Curtis, D. R., Wang, J. K., Juszczak, E., Fong, S. L., Rao, J. K. and Argos, P. (1983). The structure of bovine rhodopsin. *Biophys. Struct. Mech.* **9**, 235-244.
Hárosi, F. I. (1994). An analysis of two spectral properties of vertebrate visual pigments. *Vision Res.* **34**, 1359-1367.
Herczeg, G. and Välimäki, K. (2011). Intraspecific variation in behaviour: effects of evolutionary history, ontogenetic experience and sex. *J. Evol. Biol.* **24**, 2434-2444.
Herczeg, G., Gonda, A. and Merilä, J. (2009a). Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *J. Evol. Biol.* **22**, 544-552.
Herczeg, G., Gonda, A. and Merilä, J. (2009b). Evolution of gigantism in nine-spined sticklebacks. *Evolution* **63**, 3190-3200.
Hofmann, C. M. and Carleton, K. L. (2009). Gene duplication and differential gene expression play an important role in the diversification of visual pigments in fish. *Integr. Comp. Biol.* **49**, 630-643.
Hope, A. J., Partridge, J. C. and Hayes, P. K. (1998). Switch in rod opsin gene expression in the European eel, *Anguilla anguilla* (L.). *Proc. R. Soc. B* **265**, 869-874.
Jerlov, N. G. (1976). *Marine Optics*. Amsterdam: Elsevier.
Jokela-Määttä, M., Pahlberg, J., Lindström, M., Zak, P. P., Porter, M., Ostrovsky, M. A., Cronin, T. W. and Donner, K. (2005). Visual pigment absorbance and spectral sensitivity of the *Mysis relicta* species group (Crustacea, Mysida) in different light environments. *J. Comp. Physiol. A* **191**, 1087-1097.
Jokela-Määttä, M., Smura, T., Aaltonen, A., Ala-Laurila, P. and Donner, K. (2007). Visual pigments of Baltic Sea fishes of marine and limnic origin. *Vis. Neurosci.* **24**, 389-398.
Jokela-Määttä, M., Vartio, A., Paulin, L. and Donner, K. (2009). Individual variation in rod absorbance spectra correlated with opsin gene polymorphism in sand goby (*Pomatoschistus minutus*). *J. Exp. Biol.* **212**, 3415-3421.
Judd, D. B., MacAdam, D. L., Wyszecki, G., Budde, H. W., Condit, H. R., Henderson, S. T. and Simonds, J. L. (1964). Spectral distribution of typical daylight as a function of correlated color temperature. *J. Opt. Soc. Am.* **54**, 1031-1040.
Koli, L. (1990). *Suomen Kalat*. Porvoo, Finland: Werner Söderström Osakeyhtiö.
Koskelainen, A., Ala-Laurila, P., Fyhrquist, N. and Donner, K. (2000). Measurement of thermal contribution to photoreceptor sensitivity. *Nature* **403**, 220-223.
Larmuseau, M. H., Raeymaekers, J. A., Ruddick, K. G., Van Houdt, J. K. and Volckaert, F. A. (2009). To see in different seas: spatial variation in the rhodopsin gene of the sand goby (*Pomatoschistus minutus*). *Mol. Ecol.* **18**, 4227-4239.
Larmuseau, M. H., Vancampenhout, K., Raeymaekers, J. A., Van Houdt, J. K. and Volckaert, F. A. (2010). Differential modes of selection on the rhodopsin gene in coastal Baltic and North Sea populations of the sand goby, *Pomatoschistus minutus*. *Mol. Ecol.* **19**, 2256-2268.
Lindström, M. (2000). Seasonal changes in the underwater light milieu in a Finnish Baltic sea coastal locality. *Geophysica* **36**, 215-232.
Luo, D. G., Xue, T. and Yau, K. W. (2008). How vision begins: an odyssey. *Proc. Natl. Acad. Sci. USA* **105**, 9855-9862.
Luo, D. G., Yue, W. W., Ala-Laurila, P. and Yau, K. W. (2011). Activation of visual pigments by light and heat. *Science* **332**, 1307-1312.
Lythgoe, J. N. (1979). *The Ecology of Vision*. Oxford: Clarendon Press.
Lythgoe, J. N. (1984). Visual pigments and environmental light. *Vision Res.* **24**, 1539-1550.
Mäkelä, A. V., Heikkilä, O., Kilpeläinen, I. and Heikkinen, S. (2011). ImatraNMR: novel software for batch integration and analysis of quantitative NMR spectra. *J. Magn. Reson.* **211**, 186-194.
Nathans, J. (1990a). Determinants of visual pigment absorbance: role of charged amino acids in the putative transmembrane segments. *Biochemistry* **29**, 937-942.
Nathans, J. (1990b). Determinants of visual pigment absorbance: identification of the retinylidene Schiff's base counterion in bovine rhodopsin. *Biochemistry* **29**, 9746-9752.
Parry, J. W. and Bowmaker, J. K. (2000). Visual pigment reconstitution in intact goldfish retina using synthetic retinaldehyde isomers. *Vision Res.* **40**, 2241-2247.
Parry, J. W., Carleton, K. L., Spady, T., Carboo, A., Hunt, D. M. and Bowmaker, J. K. (2005). Mix and match color vision: tuning spectral sensitivity by differential opsin gene expression in Lake Malawi cichlids. *Curr. Biol.* **15**, 1734-1739.
Reuter, T. (1969). Visual pigments and ganglion cell activity in the retinae of tadpoles and adult frogs (*Rana temporaria* L.). *Acta Zool. Fenn.* **122**, 1-64.
Reuter, T. E., White, R. H. and Wald, G. (1971). Rhodopsin and porphyropsin fields in the adult bullfrog retina. *J. Gen. Physiol.* **58**, 351-371.
Schwanzara, S. A. (1967). The visual pigments of freshwater fishes. *Vision Res.* **7**, 121-148.
Shand, J. (1993). Changes in the spectral absorption of cone visual pigments during the settlement of the goatfish *Upeneus tragula*: the loss of red sensitivity as a benthic existence begins. *J. Comp. Physiol. A* **173**, 115-121.
Shand, J., Hart, N. S., Thomas, N. and Partridge, J. C. (2002). Developmental changes in the cone visual pigments of black bream *Acanthopagrus butcheri*. *J. Exp. Biol.* **205**, 3661-3667.
Shikano, T., Shimada, Y., Herczeg, G. and Merilä, J. (2010). History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. *Mol. Ecol.* **19**, 1147-1161.
Spady, T. C., Seehausen, O., Loew, E. R., Jordan, R. C., Kocher, T. D. and Carleton, K. L. (2005). Adaptive molecular evolution in the opsin genes of rapidly speciating cichlid species. *Mol. Biol. Evol.* **22**, 1412-1422.
Spady, T. C., Parry, J. W., Robinson, P. R., Hunt, D. M., Bowmaker, J. K. and Carleton, K. L. (2006). Evolution of the cichlid visual palette through ontogenetic subfunctionalization of the opsin gene arrays. *Mol. Biol. Evol.* **23**, 1538-1547.
Suzuki, T., Makino-Tasaka, M. and Miyata, S. (1985). Competition between retinal and 3-dehydroretinal for opsin in the regeneration of visual pigment. *Vision Res.* **25**, 149-154.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731-2739.
Temple, S. E., Plate, E. M., Ramsden, S., Haimberger, T. J., Roth, W. M. and Hawryshyn, C. W. (2006). Seasonal cycle in vitamin A1/A2-based visual pigment composition during the life history of coho salmon (*Oncorhynchus kisutch*). *J. Comp. Physiol. A* **192**, 301-313.
Terai, Y., Seehausen, O., Sasaki, T., Takahashi, K., Mizoiri, S., Sugawara, T., Sato, T., Watanabe, M., Konijnendijk, N., Mrosso, H. D. et al. (2006). Divergent selection on opsins drives incipient speciation in Lake Victoria cichlids. *PLoS Biol.* **4**, e433.
Trokovic, N., Herczeg, G., Scott McCairns, R. J., Izza Ab Ghani, N. and Merilä, J. (2011). Intraspecific divergence in the lateral line system in the nine-spined stickleback (*Pungitius pungitius*). *J. Evol. Biol.* **24**, 1546-1558.
Vorobyev, M. and Osorio, D. (1998). Receptor noise as a determinant of colour thresholds. *Proc. Biol. Sci.* **265**, 351-358.
Vorobyev, M., Brandt, R., Peitsch, D., Laughlin, S. B. and Menzel, R. (2001a). Colour thresholds and receptor noise: behaviour and physiology compared. *Vision Res.* **41**, 639-653.
Vorobyev, M., Marshall, J., Osorio, D., Hempel de Ibarra, N. and Menzel, R. (2001b). Colourful objects through animal eyes. *Color Res. Appl.* **26 Suppl. 1**, S214-S217.
Wald, G. (1937). Visual purple system in freshwater fishes. *Nature* **139**, 1017-1018.
Wald, G. (1939). On the distribution of vitamins A_1 and A_2. *J. Gen. Physiol.* **22**, 391-415.
Wald, G. (1941). The visual system of euryhaline fishes. *J. Gen. Physiol.* **25**, 235-245.
Wald, G. (1946). The metamorphosis of visual system in Amphibia. *Biol. Bull.* **91**, 239.
Wang, J. S. and Kefalov, V. J. (2011). The cone-specific visual cycle. *Prog. Retin. Eye Res.* **30**, 115-128.
Wang, J. S., Estevez, M. E., Cornwall, M. C. and Kefalov, V. J. (2009). Intra-retinal visual cycle required for rapid and complete cone dark adaptation. *Nat. Neurosci.* **12**, 295-302.
Ward, J. L., Harris, C., Lewis, J. and Beale, M. H. (2003). Assessment of 1H NMR spectroscopy and multivariate analysis as a technique for metabolite fingerprinting of *Arabidopsis thaliana*. *Phytochemistry* **62**, 949-957.
Whitmore, A. V. and Bowmaker, J. K. (1989). Seasonal variation in cone sensitivity and short-wave absorbing visual pigments in the rudd *Scardinius erythrophthalmus*. *J. Comp. Physiol. A* **166**, 103-115.
Yokoyama, S. and Yokoyama, R. (2000). Comparative molecular biology of visual pigments. In *Handbook of Biological Physics*, Vol. 3: *Molecular Mechanisms in Visual Transduction* (ed. D. G. Stavenga, W. J. DeGrip and E. N. Pugh), pp. 257-296. Amsterdam: Elsevier.

MOLECULAR ECOLOGY

Molecular Ecology (2010) 19, 1147–1161 doi: 10.1111/j.1365-294X.2010.04553.x

History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations

TAKAHITO SHIKANO, YUKINORI SHIMADA, GÁBOR HERCZEG and JUHA MERILÄ

Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, PO Box 65, FI-00014, Helsinki, Finland

Abstract

The genetic structure of contemporary populations can be shaped by both their history and current ecological conditions. We assessed the relative importance of postglacial colonization history and habitat type in the patterns and degree of genetic diversity and differentiation in northern European nine-spined sticklebacks (*Pungitius pungitius*), using mitochondrial DNA (mtDNA) sequences and 12 nuclear microsatellite and insertion/deletion loci. The mtDNA analyses identified – and microsatellite analyses supported – the existence of two historically distinct lineages (eastern and western). The analyses of nuclear loci among 51 European sites revealed clear historically influenced and to minor degree habitat dependent, patterns of genetic diversity and differentiation. While the effect of habitat type on the levels of genetic variation (coastal > freshwater) and differentiation (freshwater > coastal) was clear, the levels of genetic variability and differentiation in the freshwater sites were independent of habitat type (viz. river, lake and pond). However, levels of genetic variability, together with estimates of historical effective population sizes, decreased dramatically and linearly with increasing latitude. These geographical patterns of genetic variability and differentiation suggest that the contemporary genetic structure of freshwater nine-spined sticklebacks has been strongly impacted by the founder events associated with postglacial colonization and less by current ecological conditions (cf. habitat type). In general, the results highlight the strong and persistent effects of postglacial colonization history on genetic structuring of northern European fauna and provide an unparalleled example of latitudinal trends in levels of genetic diversity.

Keywords: bottleneck, colonization history, founder effect, genetic diversity, phylogeography, *Pungitius*

Received 30 March 2009; revision received 21 December 2009; accepted 22 December 2009

Introduction

Understanding the relative importance of historical and ecological factors that influence the genetic structure of natural populations is a topic central to contemporary evolutionary biology (Vucetich & Waite 2003; Johansson *et al.* 2006; Sagarin *et al.* 2006; Eckert *et al.* 2008). In the Northern Hemisphere, the Pleistocene climate oscillations have had a large impact on the genetic structuring of its fauna and flora (Hewitt 1996, 1999, 2004; Bernatchez & Wilson 1998; Schmitt 2007). During the last glacial maximum, most species were forced into southern refugia from which they expanded north when the climate warmed (Hewitt 1996). Since the northward expansion from southern refugia was performed by a limited number of individuals, postglacial colonization is expected to have lead to founder effects and thereby also to the loss of genetic diversity in northern populations (Hewitt 1996). Significant consequences of postglacial colonization on population genetic structure have been demonstrated in several organisms (e.g. Hewitt 1996; Merilä *et al.* 1997; Schmitt & Seitz 2002; Adams *et al.* 2006; Muller *et al.* 2008).

Correspondence: T. Shikano, Fax: 358 9 191 57694;
E-mail: takahito.shikano@helsinki.fi

In addition to colonization history, contemporary genetic population structure can be strongly influenced by ecological factors through their effects on gene flow, genetic drift and selection. For instance, habitat type is known to be an important determinant of genetic diversity within and among fish populations (Gyllensten 1985; Ward *et al.* 1994; DeWoody & Avise 2000). Open marine habitats can sustain large effective population sizes and facilitate gene flow among populations over large geographic distances. In contrast, physical isolation of freshwater habitats prevents gene flow between localities and leads to substantial genetic subdivision among populations. As a result, freshwater fish generally show higher degree of genetic differentiation than marine fish (Gyllensten 1985; Ward *et al.* 1994). In addition, freshwater fish populations confined to particular lakes or river drainages for long periods of time tend to have lower levels of genetic variation than marine populations due to their smaller effective population sizes (e.g. Gyllensten 1985; Ward *et al.* 1994; Tonteri *et al.* 2007).

The nine-spined stickleback (*Pungitius pungitius*) is a cold-water adapted fish having a circumpolar distribution in the Northern Hemisphere and is found in a wide variety of habitats (Wootton 1976). In Europe, nine-spined sticklebacks are distributed primarily in freshwater environments in the north, but they occur also in saline environments along coastal areas of the Arctic Ocean, the White Sea and the Baltic Sea (Paepke 2001). As northern Europe was covered by the continental ice sheet until approximately 12 000 years ago, nine-spined sticklebacks of this area should originate from ancestors formerly resident in non-glaciated areas. In Fennoscandia, postglacial colonization by terrestrial organisms occurred typically from the east and/or from the south (Hewitt 1999; Pamilo & Savolainen 1999), but colonization patterns of fish fauna are more variable (reviewed in Makhrov & Bolotov 2006).

In the case of the three-spined stickleback (*Gasterosteus aculeatus*) – a sister species to the nine-spined stickleback – freshwater habitats of northern Europe were colonized by ancestral marine fish (Mäkinen *et al.* 2006; Mäkinen & Merilä 2008). As these stickleback species exhibit similar ecological and morphological characteristics (Bell & Foster 1994), comparison of their colonization histories and population structures in the same area is of particular interest. Fennoscandia is especially interesting in this perspective since it was colonized by these species relatively recently, most likely after the last glacial maximum. Furthermore, in contrast to their restricted occurrence in coastal habitats elsewhere in the world (McPhail 1963; Takata *et al.* 1987; Paepke 2001), nine-spined sticklebacks are common in coastal sites of Fennoscandia. In eastern Asia, coastal nine-spined sticklebacks are genetically so divergent from freshwater conspecifics that they can be considered as biologically different species (Takata *et al.* 1987). Yet, little is known about the evolutionary history and genetic population structure of nine-spined sticklebacks in Europe (but see Haglund *et al.* 1992).

The main aim of this study was to investigate the phylogenetic relationships and genetic population structure of the nine-spined stickleback in northern Europe. In particular, we were interested in assessing the relative importance of postglacial colonization history and habitat type (viz. coastal, river, lake and pond) in the levels of genetic variability within sites, as well as in the degree of genetic differentiation among sites. We hypothesized that if northern European nine-spined sticklebacks have colonized from freshwater refugia and experienced severe bottlenecks during this process (cf. Hewitt 1996), strong geographically (e.g. latitudinally) ordered effects can be found in contemporary population genetic structure. For instance, the estimates of genetic variability and historical effective population size should decline towards the edge of colonization range independent of habitat type. In contrast, if ecological factors override historical factors, habitat-type effects should be apparent in the patterns of genetic variability and differentiation. For instance, one would expect to see genetic variability to be highest in coastal populations, lower in river and lake populations and lowest in small and isolated pond populations. We tested these expectations by analysing variation in mitochondrial DNA (mtDNA) and 11 microsatellite and one insertion/deletion loci in a large number (51) of sites. Large-scale phylogeographic patterns were investigated using mtDNA cytochrome *b* sequence data from 25 sites, followed by analyses of population genetic structure using nuclear loci. The results were contrasted with those from ecologically and morphologically similar three-spined sticklebacks inhabiting the same area.

Materials and methods

Samples

A total of 1754 adult or juvenile fish were collected with seine nets, minnow traps or electric fishing from 51 locations in Europe and one river in Japan during 2002–2008 (Fig. 1, Table S1, Supporting Information). The average sample size per site was 34 (range = 3–84) individuals (Table S1, Supporting Information). The fish or fin clips were stored in 70–99% ethanol for DNA extraction. Sampling sites were classified according to

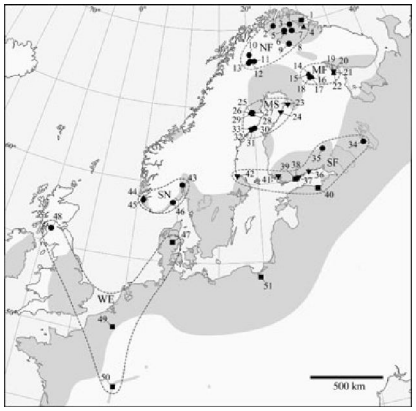


Fig. 1 Location of 51 European nine-spined stickleback sampling sites. Inverted triangle: coastal, circle: lake, square: river, triangle: pond. NF = northern Finland, MF = middle Finland, MS = middle Sweden, SF = southern Finland, SN = southern Norway, WE = Western Europe. For abbreviations of site identity codes, see Table S1 (Supporting information). Gray shading indicates geographical distribution of nine-spined sticklebacks in Europe according to Paepke (2001).

their habitat type as coastal (n = 10), river (n = 8), lake (n = 19) and pond (n = 14) sites (Table S1, Supporting Information). For a fine scale analysis with nuclear markers, European samples were categorized geographically into six groups: northern Finnish (NF), middle Finnish (MF), middle Swedish (MS), southern Finnish (SF), southern Norwegian (SN) and western European (WE; Fig. 1) sites.

mtDNA sequencing and nuclear marker genotyping

Total DNA was extracted from fin clips with a silica-fine based purification (Elphinstone *et al.* 2003) or a phenol–chloroform method (Taggart *et al.* 1992) following proteinase K digestion. A total of 96 individuals from 25 sites covering a wide geographic range were used for cytochrome *b* sequencing. Two slightly overlapping regions of cytochrome *b* gene were amplified using primers L14724 (Kocher *et al.* 1989) and PP14959 (this study; 5′-TGGTGGAGGAAMAGAAGGTG-3′), as well as PP14896 (this study; 5′-CTAACCC-GATTCTTTGCCTT-3′) and CB6Thr (Palumbi 1996). As PCR amplification was not obtained for the latter region in a few individuals, PP15469 (this study; 5′-CTATTGCTGCTCCTGGGTAA-3′) instead of CB6Thr was used for them. The newly designed primers were developed based on conserved regions of the cytochrome *b* sequences of *Pungitius pungitius* (Mattern 2004). PCR amplifications were carried out in 15 μL reaction volumes consisting of 1 × PCR buffer (Bioline), 1.5 mM $MgCl_2$, 0.25 mM dNTP (Finnzymes), 0.15 U BIOTAQ DNA polymerase (Bioline), 5 pmol of each primer and approximately 20 ng of genomic DNA. The reactions were performed as follows: an initial degeneration step at 95 °C for 2 min, followed by 30 s at 95 °C, 30 s at 52 °C and 30 s at 72 °C for 35 cycles with a final extension at 72 °C for 10 min. PCR products were purified using exonuclease I (New England Biolabs) and shrimp alkaline phosphatase (Roche) and directly sequenced in both forward and reverse directions with the same primers as those used in the PCRs. The sequencing reactions were performed in 10 μL volumes using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to manufacture's instructions. Cycle sequencing products were purified by the Montage SEQ_{96} Sequencing Reaction Cleaning Kit (Millipore) and analysed on a MegaBACE 1000 automated sequencer (Amersham Biosciences).

Allelic variation in nuclear markers was assessed using 11 microsatellite loci, Gac1125PBBE, Gac4174PBBE, Gac7033PBBE (Largiadèr *et al.* 1999; Mäkinen *et al.* 2007), Stn49, Stn96, Stn100, Stn130, Stn163, Stn173, Stn196 and Stn198 (Peichel *et al.* 2001; Mäkinen *et al.* 2007) and an insertion/deletion locus, Stn380 (Colosimo *et al.* 2005). Each forward primer was labelled with a fluorescent dye (FAM, HEX or TET) for visualization of PCR products, and the 5′-end of each reverse primer was modified with a GTTT-tail to enhance the 3′-adenylation (Brownstein *et al.* 1996). The 12 loci were arranged in multiplex PCR panels with non-overlapping size ranges in each dye. PCRs were carried out using the Qiagen Multiplex PCR Kit (Qiagen) in 10 μL reaction volumes containing 1 × Qiagen Multiplex PCR Master Mix, 0.5 × Q-Solution, 2 pmol of each primer and 10–20 ng of template DNA. The reactions for all loci were performed by the following cycle: an initial activation step at 95 °C for 15 min, followed by 30 s at 94 °C, 90 s at 53 °C and 60 s at 72 °C for 30 cycles with a final extension at 60 °C for 5 min. PCR products were visualized with a MegaBACE 1000 automated sequencer (Amersham Biosciences) and their sizes were determined with ET-ROX 550 size standard (Amersham Biosciences). Alleles were scored using Fragment Profiler 1.2 (Amersham Biosciences) with visual inspection and manual corrections of alleles.

Data analyses of mtDNA

The two regions of cytochrome *b* gene sequenced were aligned using MEGA 4 (Tamura *et al.* 2007) and combined

to obtain a 1104 base pair (bp) sequence, which corresponds to 96.8% of the gene. Sequences were deposited in GenBank under accession numbers GU227740–GU227783. Number of haplotypes, haplotype diversity (h), average number of nucleotide differences (k) and nucleotide diversity (π) were calculated using DnaSP 4.1 (Rozas *et al.* 2003). Phylogenetic analyses were conducted using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). A MrBayes setting for the best fit model (GTR + G) was selected by the hierarchical likelihood ratio tests with MrModeltest 2.3 (Nylander 2004) in conjunction with PAUP 4.0b10 (Swofford 2002). Markov chains were run for 3×10^6 generations (the average SD of split frequencies <0.01) with four chains starting from a random tree. Sampling frequency was set at 100 generations and the first 7500 samples (25%) were excluded as burn-in. The phylogenetic tree was rooted using Japanese nine-spined sticklebacks, as they are genetically divergent from European nine-spined sticklebacks (Haglund *et al.* 1992).

Data analyses of nuclear markers

Locus and population (sampling site) specific gene diversities (H_E, Nei 1987) were estimated using FSTAT 2.9.3 (Goudet 2001). Allelic richness was estimated using a rarefaction procedure in HP-RARE 1.0 (Kalinowski 2005) with a rarefaction sample size of six genes. Comparison of the estimates of allelic richness with rarefaction sample sizes of six and 20 genes using sites with more than 10 individuals confirmed that estimates based on different rarefaction sample sizes were virtually identical (r = 0.981), justifying the inclusion of sites with small sample sizes into the analyses. The presence of null alleles was tested using MICRO-CHECKER (Van Oosterhout *et al.* 2004). Within population and locus specific F_{IS} were estimated (10 000 permutations) for each site to detect possible deviations from Hardy–Weinberg equilibrium. A linkage disequilibrium test was performed between all loci over all samples. The degree of population differentiation was quantified using the standardized variance in allele frequencies (F_{ST}) as estimated by θ (Weir & Cockerham 1984). Standard errors of F_{ST} were obtained by jackknifing over loci and significance tests were performed by 1000 permutations. The genetic parameters and significance tests were estimated using FSTAT 2.9.3.

Genetic signatures of recent population size reductions were searched for by using the approach of Cornuet & Luikart (1996) with the two-phase model (Di Rienzo *et al.* 1994), as implemented in BOTTLENECK (Piry *et al.* 1999). The parameters were set to 90% stepwise mutations and 10% multistep mutations with a variance among multiple steps of 12, as recommended by Piry *et al.* (1999). This analysis tests for a relative heterozygote excess that is apparent for a few generations after a population bottleneck. The significance of heterozygote excess was assessed by the Wilcoxon signed rank test with 10 000 iterations. In addition, historical effective population sizes and migration rates were estimated using the coalescent-based maximum-likelihood method implemented in MIGRATE 3.0.3 (Beerli & Felsenstein 2001). Theta ($\theta = 4N_e\mu$, where N_e is effective population size and μ mutation rate) and the migration parameter M (m/μ, where m is migration rate) were calculated simultaneously under a stepwise mutation model with a Markov chain Monte Carlo (MCMC) repetition of 20 short chains of 20 000 steps and three long chains of 200 000 steps. F_{ST}-based estimates were used as the starting parameters and the burn-in was set to 10 000. We applied the Gelman's convergence criterion to extend the long chains until the criterion was satisfied. To ensure convergence, we repeated the analysis twice and confirmed that results were consistent between independent runs. The analyses were performed within the six geographically categorized areas (Fig. 1, Table S1, Supporting Information) to minimize the effects of 'ghost' populations (Beerli 2004). Since no significant differences in the migration parameter M were observed among freshwater habitat types (river, lake and pond) or between coastal-to-freshwater and freshwater-to-coastal sites (ANOVA or paired t-tests, $P > 0.05$), we combined the data and compared the estimates within freshwater or coastal sites, as well as among them in the six areas (cf. Fig. 1). We also note that since three loci (Stn49, Stn198 and Stn380) did not follow a stepwise mutation pattern due to an insertion of 1 bp variation, these loci were excluded from the analyses conducted with BOTTLENECK and MIGRATE.

Hierarchical genetic structuring was analysed by assessing the relative contribution of among group, among site and within site components for partition of total molecular variance (AMOVA, Excoffier *et al.* 1992) using Arlequin 3.11 (Excoffier *et al.* 2005). Significances of different hierarchical levels were tested with 1000 permutations. The contributions of mtDNA lineage (see 'Results'), habitat type (Table S1, Supporting Information) and geographical area (Table S1, Supporting Information) were analysed in all European sites and also separately for all European freshwater sites.

Genetic relationships among sites were investigated by using D_A distances (Nei *et al.* 1983), which have proven to be useful for reconstructing phylogenies (Takezaki & Nei 1996). A neighbour-joining (NJ) tree constructed from the D_A matrix was used to visualize the relationships by bootstrapping (1000 replicates) across loci to test the stability of the tree-branching pattern using Populations 1.2 (Langella 2002). Japanese

nine-spined sticklebacks were used as the outgroup. In addition to a NJ tree, the pattern of population differentiation was investigated using a two-dimensional scaling plot of D_A distances with SPSS 13.0 (SPSS Inc.). To further evaluate the genetic relationships, allele frequencies were subjected to principal component analysis (PCA) based on the correlation matrix of allele frequencies between sites. PCA was performed using PCA-GEN 1.2 (www2.unil.ch/popgen/softwares/pcagen.htm) with 10 000 randomizations. We also applied a Bayesian approach for population structure estimation using STRUCTURE 2.2 (Pritchard *et al.* 2000). Assuming that each individual comes from one of the K populations, a no-admixture model of correlated allele frequencies was run with 50 000 burn-in length periods and 100 000 MCMC repetitions, with values of K = 2–20 and five parallel chains for each K. While ΔK (Evanno *et al.* 2005) has been suggested to be useful to determine K, a clear peak of ΔK was not found in our analyses. As the log likelihood reached the maximum at $K = 8$ and the variance increased at $K \geq 9$ (data not shown), we used $K = 8$ in the further analyses.

Sequential Bonferroni corrections (Rice 1989) were applied for all multiple comparisons to minimize type I errors. Only an infinite-allele model was used for population differentiation and phylogeny analyses, as this model is considered more reliable than a stepwise mutation model for phylogenetic analyses of bottlenecked populations (as in our case; see below; Takezaki & Nei 1996; Tomiuk *et al.* 1998). We also note that there were no significant differences in observed allele number, allelic richness, heterozygosity and F_{ST} between microsatellite and insertion/deletion loci (Mann–Whitney *U*-tests, $P > 0.05$ in all comparisons). In addition, inclusion or exclusion of the insertion/deletion locus did not qualitatively affect neither population comparisons nor phylogenetic relationships (data not shown). Hence, microsatellite and insertion/deletion data were combined for population comparisons and phylogenetic analyses.

Geographic trend tests at nuclear markers

The impact of postglacial population expansion on genetic variation and differentiation in nuclear markers was investigated by inspecting patterns in these measures, in respect to latitude and longitude, within the lineages identified by cytochrome *b* sequences and nuclear markers (see 'Results'). These analyses were performed separately using data from (i) all sites; and (ii) freshwater sites, as high rates of gene flow/migration were expected among the coastal sites (see 'Results').

A rapid postglacial population expansion would be expected to appear as a negative trend in diversity measures towards the direction of the expansion (Hewitt 1996, 1999). This was tested by regressing intrapopulation genetic variability measures (allelic richness and heterozygosity) against latitude and longitude. Prior to this test, the effects of different habitat types on levels of genetic variation were evaluated, since current levels of genetic variability can also be affected by habitat type if they differ systematically in their effective population sizes. This was done by using general linear models (GLMs) where allelic richness or heterozygosity was treated as a dependent variable, lineage and habitat type as factors and latitude and longitude as covariates.

Isolation by distance was tested by correlating the genetic distance measures (F_{ST} and D_A) with geographic distances between sites. To investigate population expansion patterns, we evaluated latitudinal and longitudinal trends in different genetic differentiation parameters (F_{ST}, D_A, dimensional measures of D_A and principal component scores). A stronger association with latitudinal than longitudinal distances would be expected if a population expansion trend is in a latitudinal direction (e.g. northward expansion) and vice versa. This trend was tested by using correlations between pairwise matrices of F_{ST} or D_A and latitudinal or longitudinal differences among sites. To further evaluate genetic differentiation patterns, the dimensional measures of D_A or principal component scores were correlated with latitudinal and longitudinal coordinates. Statistical significance of (Pearson) correlations was evaluated by Mantel tests using Isolde (in Genepop, Rousset 2008) with 1000 permutations in matrix correlations.

Results

Mitochondrial DNA phylogeny

The 1104 bp fragment of cytochrome *b* contained 50 polymorphic sites defining 39 haplotypes in the 90 European individuals sequenced (haplotype diversity = 0.890; Table S2, Supporting Information). The overall nucleotide diversity (π) and average number of nucleotide differences (k) were 0.0051 and 5.637, respectively. The most common haplotype (E3) was found in 29 individuals and widely distributed in Fennoscandia (14 out of 20 sites; Table S1, Supporting Information).

The Bayesian phylogeny was constructed using the 39 European haplotypes and rooted by the five Japanese haplotypes identified in a river site. The European mtDNA haplotypes clustered into distinct eastern (E1–27, E39) and western (E28–38) lineages supported by a high posterior probability (1.00; Fig. 2). The eastern lineage consists of all the haplotypes collected in the Fennoscandian (NF, MF, MS and SF) sites except for

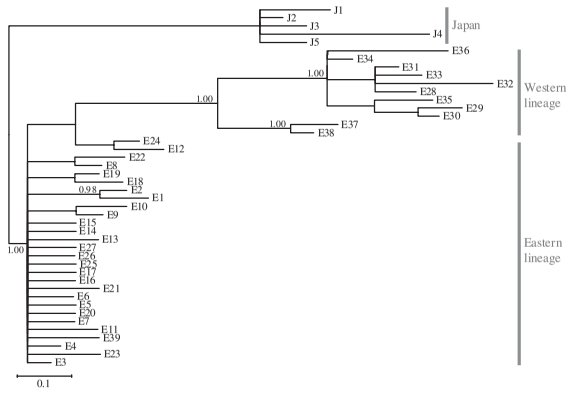


Fig. 2 Bayesian phylogeny of 39 cytochrome *b* haplotypes in European nine-spined sticklebacks. Posterior probabilities >0.95 are indicated.

the SN sites. This lineage also includes the haplotype (E39) found at a Polish site (PL-PAS). Accordingly, the most common haplotype (E3) was shared by 44.6% of the individuals in the eastern lineage. The western lineage consists of all the haplotypes found in the WE and SN sites. Within this lineage, two subgroups of haplotypes were identified with a high posterior probability (1.00), one of which consists of the haplotypes (E37–38) found at a French site (FR-MON; Fig. 2).

Within each main lineage, haplotype diversity was lower in the eastern lineage (h = 0.799, SD = 0.052) than in the western lineage (h = 0.923, SD = 0.025). Similarly, the eastern lineage exhibited lower nucleotide diversity (π = 0.00147 vs. 0.00428) and k (k = 1.620 vs. 4.727) as compared to the western lineage. The same trend was obtained even if the French samples were excluded from the analyses of the western lineage (h = 0.908, π = 0.00270, k = 2.980).

Based on these results (see also Fig. 4), samples from NF, MF, MS and SF sites were treated as the eastern lineage, and those from SN and WE sites as the western lineage for the remainder of this paper.

Genetic variability at nuclear loci

All nuclear loci were amplified successfully in all sites except in PL-PAS where Gac4174PBBE and Gac7033PBBE completely failed even after retrials. Hence, this site was excluded from further analyses. A total of 280 alleles were observed in European samples across the 12 loci with an average of 23.3 alleles per locus (range = 7–90). No linkage disequilibrium was detected between the 12 loci. MICRO-CHECKER analyses did not indicate the presence of null alleles, with the possible exceptions of the locus Stn163 in the RU-LEV, the locus Gac4174PBBE in the SE-NAV and the loci Stn100, Stn130, Stn163 and Stn198 in the NO-OXN. Coupled with the indication of possible null alleles at multiple loci, a relatively high F_{IS} (0.123) in the NO-OXN suggested the effect of inbreeding rather than null alleles. There was no evidence for deviations from Hardy-Weinberg equilibrium at any locus in any of the sites. BOTTLENECK tests did not reveal any evidence for recent population size bottlenecks in any of the sites. In addition, F_{IS} values were homogenous across different habitat types (Table S1, Supporting Information).

Levels of genetic variability – whether measured by allelic richness or expected heterozygosity – varied significantly among sites (Table S1, Supporting Information). Allelic richness (±SE) within sites varied from 1.01 (±0.01) to 2.94 (±0.28) and heterozygosity from 0.004 (±0.003) to 0.604 (±0.063). Genetic variability was very low and close to zero in the FI-PYO (Table S1, Supporting Information). On average, the freshwater sites exhibited significantly lower allelic richness (1.96 vs. 2.81) and heterozygosities (0.332 vs. 0.578) than the coastal sites (Mann–Whitney *U*-tests, $P < 0.001$). In the freshwater sites, genetic variation was generally lower in northern and mid-Fennoscandia as compared to the southern areas, although relatively high levels of genetic variation were observed also in the three Russian pond sites (RU-MAS, BOL and KRU) and the Swedish lake site (SE-KRO), which are all closely located to the sea (Fig. 1, Table S1, Supporting Information). A hierarchical analysis of genetic variation indicated significant effects of habitat type, latitude and longitude on both allelic richness and heterozygosity (Table 1). However, once the analysis was restricted to

the freshwater sites, the effect of habitat type was no longer significant (Table 1). Therefore, the effect of habitat type appears to be strongly influenced by the difference between coastal and freshwater sites.

In the eastern lineage, both allelic richness ($r^2 = 0.440$, $n = 42$, $P < 0.001$) and heterozygosity ($r^2 = 0.455$, $n = 42$, $P < 0.001$) decreased with increasing latitude (Fig. 3A and B). These trends were significant even when the analyses was restricted to the freshwater sites (allelic richness, $r^2 = 0.359$, $n = 32$, $P < 0.001$; heterozygosity, $r^2 = 0.358$, $n = 32$, $P < 0.001$). In contrast, no significant longitudinal trends were found among all sites or among the freshwater sites of this lineage ($P > 0.05$). In the western lineage, allelic richness and heterozygosity were unrelated to latitude or longitude ($P > 0.05$; Fig. 3A and B).

The estimates of historical effective population size as represented by theta varied from 0.17 to 11.35 among the sites and relatively high values were observed in southwestern Europe (area WE; Table S1, Supporting Information). Assuming a microsatellite mutation rate of 5×10^{-4} (Estoup & Angers 1998; Lippe *et al.* 2006), these correspond to effective population sizes of N_e = 86–5674. A hierarchical analysis of theta indicated significant effects of lineage and latitude (Table 1). Within lineages, theta decreased significantly with increasing latitude (Fig. 3C; eastern lineage, $r^2 = 0.247$, $n = 42$, $P < 0.001$; western lineage, $r^2 = 0.607$, $n = 8$, $P < 0.05$), while no significant correlation was observed between longitude and theta ($P > 0.05$). The same trends were obtained in the analyses of freshwater sites of the eastern lineage (latitude, $r^2 = 0.311$, $n = 32$, $P < 0.001$; longitude, $P > 0.05$).

The effects of geographic area and habitat type on the estimates of the migration parameter M were significant (GLMs, $P < 0.001$). In the comparison of freshwater sites, the average value was relatively high in the NF, SF and SN sites as compared to other areas (Table S3, Supporting Information). In the MS and SF sites, the estimate was higher among coastal sites as compared to that among freshwater sites (Table S3, Supporting Information).

Genetic differentiation at nuclear loci

The degree of genetic differentiation among all European sites was very high with an average $F_{ST} = 0.415$ (SE = 0.031, $P < 0.001$). The degree of differentiation among the coastal sites was low ($F_{ST} = 0.038 \pm 0.009$, $P < 0.001$), especially within the Baltic Sea ($F_{ST} = 0.011 \pm 0.003$, $P < 0.001$). In contrast, freshwater sites were more differentiated than coastal sites and the degree of genetic differentiation was high in Europe ($F_{ST} = 0.492 \pm 0.034$, $P < 0.001$) and also within the eastern lineage ($F_{ST} = 0.494 \pm 0.035$, $P < 0.001$).

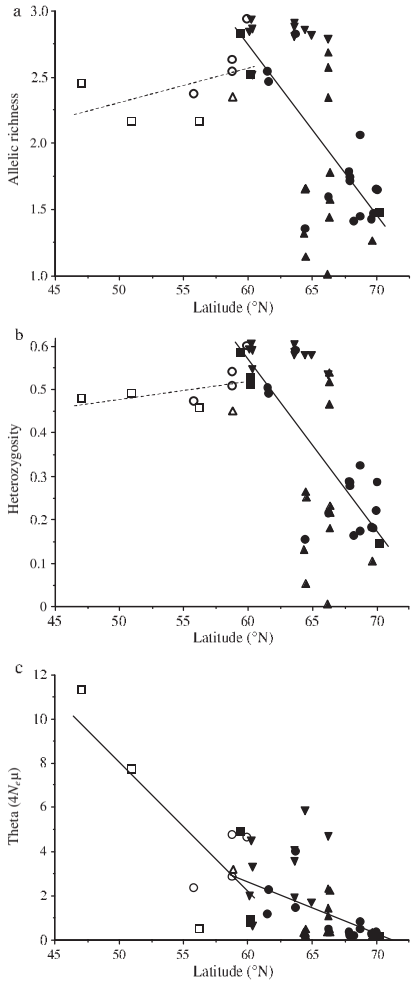


Fig. 3 Relationship between latitude and allelic richness (a), heterozygosity (b) or theta (c) in European nine-spined sticklebacks. Inverted triangle: coastal, circle: lake, square: river, triangle: pond. Eastern lineage (closed symbols), $r^2 = 0.440$, $n = 42$, $P < 0.001$ (A), $r^2 = 0.455$, $n = 42$, $P < 0.001$ (B), $r^2 = 0.247$, $n = 42$, $P < 0.001$ (C); western lineage (open symbols), $r^2 = 0.213$, $n = 8$, $P = 0.250$ (A), $r^2 = 0.146$, $n = 8$, $P = 0.350$ (B), $r^2 = 0.607$, $n = 8$, $P = 0.023$ (C).

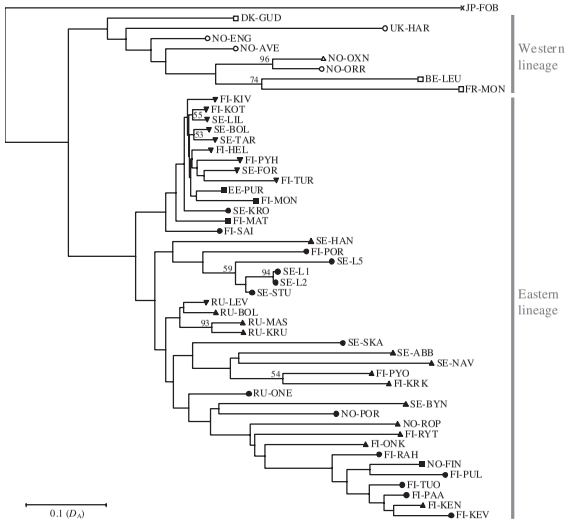


Fig. 4 A neighbour-joining tree based on nuclear markers for 50 European nine-spined stickleback sites. Inverted triangle: coastal, circle: lake, square: river, triangle: pond. Closed symbols = eastern lineage, open symbols = western lineage, x = Japanese sample. Bootstrap supports >50% are indicated.

Table 1 Hierarchical analysis of genetic variation and theta in European nine-spined sticklebacks

	All sites ($n = 50$)					Freshwater sites ($n = 40$)				
Estimate Source	Type III SS	df	MS	F	P	Type III SS	df	MS	F	P
Allelic richness										
Lineage	0.443	1	0.443	3.074	0.087	0.434	1	0.434	2.626	0.114
Habitat type	5.357	3	1.786	12.381	0.000	0.894	2	0.447	2.704	0.081
Latitude	1.836	1	1.836	12.730	0.001	2.116	1	2.116	12.792	0.001
Longitude	1.265	1	1.265	8.774	0.005	1.504	1	1.504	9.096	0.005
Error	6.202	43	0.144			5.623	34	0.165		
Expected heterozygosity										
Lineage	0.033	1	0.033	2.512	0.120	0.035	1	0.035	2.283	0.140
Habitat type	0.437	3	0.146	11.034	0.000	0.085	2	0.042	2.738	0.079
Latitude	0.189	1	0.189	14.309	0.000	0.207	1	0.207	13.335	0.001
Longitude	0.096	1	0.096	7.260	0.010	0.119	1	0.119	7.665	0.009
Error	0.568	43	0.013			0.527	34	0.016		
Theta ($4N_e\mu$)										
Lineage	12.025	1	12.025	5.490	0.024	6.958	1	6.958	3.881	0.057
Habitat type	18.246	3	6.082	2.777	0.053	0.684	2	0.342	0.191	0.827
Latitude	22.809	1	22.809	10.413	0.002	32.681	1	32.681	18.227	0.000
Longitude	8.241	1	8.241	3.762	0.059	8.258	1	8.258	4.606	0.039
Error	94.185	43	2.190			60.962	34	1.793		

AMOVA analyses revealed that 49.1–58.0% of the total genetic variance resided within sites (Table 2). A significant proportion of the variance resided between lineages (19.6%, $P < 0.001$) or among geographical areas (11.1%, $P < 0.001$), but habitat type explained only a very low proportion of the variance (3.7%, $P < 0.001$). When the analysis was restricted to freshwater sites, the contribution of habitat type was not significant

Table 2 Hierarchical analysis of genetic divergence in European nine-spined sticklebacks

Group	No. of groups (sites)	Among groups			Among sites			Within sites		
		%	Φ_{CT}	*P*	%	Φ_{ST}	*P*	%	Φ_{SC}	*P*
Lineage	2 (50)	19.6	0.196	***	31.3	0.510	***	49.1	0.390	***
Eastern lineage	1 (42)				40.7	0.407	***	59.3	0.593	
Western lineage	1 (8)				24.0	0.240	***	76.0	0.760	
Geography	7 (50)	11.1	0.111	***	31.3	0.427	***	57.3	0.355	***
NF	1 (13)				51.2	0.512	***	48.8	0.488	
MF	1 (9)				44.0	0.440	***	56.0	0.560	
MS	1 (11)				39.7	0.397	***	60.3	0.603	
SF	1 (9)				7.0	0.070	***	93.0	0.930	
SN	1 (4)				12.5	0.125	***	87.5	0.875	
WE	1 (4)				34.9	0.349	***	65.1	0.651	
Habitat type (all)	4 (50)	3.7	0.037	***	38.3	0.420	***	58.0	0.398	***
Coastal	1 (10)				3.8	0.038	***	96.2	0.962	
River	1 (7)				25.5	0.255	***	74.5	0.745	
Lake	1 (19)				44.8	0.448	***	55.2	0.552	
Pond	1 (14)				58.3	0.583	***	41.7	0.417	
Habitat type (FW)	3 (40)	1.7	0.017	NS	47.7	0.494	***	50.6	0.486	***

For abbreviations of geographic regions, see Fig. 1. FW, freshwater sites. NS, not significant; *** $P < 0.001$.

(Table 2). Within lineages, an average F_{ST} was higher in the eastern lineage (0.407) than in the western lineage (0.240; Table 2). Similarly, the degree of genetic differentiation varied among geographical areas (Table 2). An average F_{ST} was relatively low in southern Fennoscandia (0.070–0.125), but higher in the mid-latitude and northern areas (0.397–0.512).

In the NJ tree obtained from D_A distances, the root for the European samples was located in the branch separating the western and eastern lineages identified by the mtDNA analysis (Fig. 4). Although bootstrap support for basal nodes was low, the topology of tree suggests clustering of sites within the two main lineages roughly according to their geographic proximity. The samples of SN sites clustered together with those of WE sites belonging to the same lineage (Fig. 4). In the eastern lineage, two main branches were apparent. One included the Baltic Sea sites and freshwater sites from southern and mid parts of Fennoscandia, the other included the White Sea and mid- and northern Fennoscandian freshwater sites (Fig. 4). Within the eastern lineage, the branches including Finnish and Swedish pond and lake sites exhibited higher degrees of genetic divergence (cf. branch lengths) than others (Fig. 4). In a dimensional analysis of D_A distances, the dimension one clearly discriminated the two lineages (Fig. S1A, Supporting Information). Rather than clusters, continuous variation along the respective dimensions was observed among sites within each lineage (Fig. S1A, Supporting Information).

In a principal component analysis, the first two principal components were significant ($P < 0.05$) and explained 35.1% of the variation in allele frequencies (Fig. S1B, Supporting Information). A combination of the two axes revealed three clusters: one cluster for the western lineage and two clusters for the eastern lineage (Fig. S1B, Supporting Information). In the latter, one corresponds to part of the NF sites and the other consists of other Fennoscandian sites.

The Bayesian analysis with the program STRUCTURE assigned the majority of individuals from each site to the same cluster, except for the FI-SAI, in which individuals were assigned mainly to two clusters (Table S1, Supporting Information). While the individuals of the western lineage were assigned to a single cluster, those of the eastern lineage were divided into seven clusters (Table S1, Supporting Information). One large cluster consisted of individuals from most SF sites and several MS sites. Each of other clusters consisted of individuals from geographically close sites. For instance, one cluster contained individuals exclusively from some MF sites. Likewise, two clusters consisted of individuals from most NF sites according to their geographic proximity. In coastal sites, the individuals of the Baltic Sea were assigned to a single cluster whereas those of the White Sea were assigned to a different cluster.

Geographic trends in genetic differentiation at nuclear loci

A significant isolation by distance effect was found in the western lineage (F_{ST}, $r^2 = 0.409$, $n = 28$, $P = 0.020$; D_A, $r^2 = 0.636$, $n = 28$, $P = 0.001$; Table 3). In the eastern lineage, D_A distances were significantly, but weakly, correlated with geographic distances across all sites ($r^2 = 0.090$, $n = 861$, $P < 0.001$), but no isolation by distance was identified when the analysis was restricted to the freshwater sites ($P > 0.05$).

Table 3 Association between genetic differentiation measures and geographic variables. Distance refers to geographic distances separating pairs of sites, latitude and longitude to coordinate differences separating pairs of sites (F_{ST} and D_A) or respective coordinates (DM and PC). Values refer Pearson correlation coefficients and number of comparisons is given in parentheses. Significance levels determined with Mantel's test (F_{ST} and D_A) or with standard statistical tests (DM and PC).

Comparison	Eastern lineage		Western lineage
	All	Freshwater	All
Distance			
F_{ST}	0.000 (861)	0.013 (496)	0.409 (28)*
D_A	0.090 (861)***	0.044 (496)	0.636 (28)**
Latitude			
F_{ST}	0.001 (861)	0.012 (496)	0.205 (28)
D_A	0.098 (861)***	0.039 (496)*	0.382 (28)*
DM1	0.530 (42)***	0.424 (32)***	0.519 (8)*
DM2	0.000 (42)	0.013 (32)	0.818 (8)**
PC1	0.565 (42)***	0.474 (32)***	0.655 (8)*
PC2	0.016 (42)	0.069 (32)	0.323 (8)
Longitude			
F_{ST}	0.001 (861)	0.000 (496)	0.218 (28)*
D_A	0.008 (861)	0.007 (496)	0.227 (28)*
DM1	0.100 (42)*	0.043 (32)	0.442 (8)
DM2	0.121 (42)*	0.220 (32)**	0.170 (8)
PC1	0.151 (42)*	0.087 (32)	0.224 (8)
PC2	0.040 (42)	0.087 (32)	0.006 (8)

DM, dimentional measure of D_A; PC, principal component score.
* $P < 0.05$, $P < 0.01$, *** $P < 0.001$.

Geographical trends in genetic differentiation were better explained in terms of latitude than longitude (Table 3). For instance, D_A distances correlated significantly with latitudinal differences between sites in the eastern lineage ($r^2 = 0.098$, $n = 861$, $P < 0.001$), with no significant associations to longitudinal differences ($P > 0.05$). The same trend was observed among the freshwater sites of this lineage (Table 3). Moreover, geographical trend tests using the dimensional measures of D_A (DM) and the principal component scores (PC) revealed that DM1 and PC1 exhibited strong latitudinal clines across all sites (DM1, $r^2 = 0.530$, $n = 42$, $P < 0.001$; PC1, $r^2 = 0.565$, $n = 42$, $P < 0.001$) as well as across the freshwater sites (DM1, $r^2 = 0.424$, $n = 32$, $P < 0.001$; PC1, $r^2 = 0.474$, $n = 32$, $P < 0.001$). In contrast, no or weak associations were detected in respect to longitude (Table 3). Similarly, the geographic patterns of population differentiation depended more on latitude than longitude in the western lineage (Table 3).

Discussion

Our results suggest a strong historical component to the patterns of genetic variation within and among northern European nine-spined stickleback sites. These historical effects appear to override the impact of contemporary ecological factors – as reflected in habitat effects – as determinants of genetic variability patterns. Firstly, two divergent groups or clades of populations (western and eastern lineages) were found. Secondly, although there was a clear effect of habitat type on genetic diversity and the degree of genetic differentiation within the eastern lineage, this effect was confined to the difference between coastal and freshwater sites. However, the levels of genetic variation within the freshwater sites were independent of habitat type, but strongly impacted by historical factors as reflected in the significant effects of latitude on the levels of genetic variability within sites. The strong decline in the levels of genetic diversity within sites as a function of latitude in the eastern lineage suggests that the Fennoscandian nine-spined sticklebacks have suffered from the reduction of genetic variation due to founder effects associated with the postglacial recolonization process. While similar examples of latitudinal decline in the levels of genetic variability are available from several terrestrial species (e.g. Hewitt 1996; Merilä *et al.* 1997; Palo *et al.* 2004), we are not aware that this clear pattern would have been described earlier for any freshwater taxa. The strong influence of population history on genetic variability highlights the long lasting influence of Pleistocene events on genetic structuring of northern European fauna (Hewitt 2000). In what follows, we will discuss these and related issues in light of the results obtained.

Phylogeography

The mtDNA analyses identified two major lineages of nine-spined sticklebacks in northern Europe: one occupying the eastern and northern parts of Fennoscandia and the other occurring in southern Norway and southwestern continental Europe. The observed geographic distribution of the two distinct lineages is similar to that typically found among terrestrial animal and plant taxa (reviewed in Hewitt 2000). While the recolonization routes of different lineages of fishes to Fennoscandia are more heterogeneous and complex than those of terrestrial organisms (reviewed in Makhrov & Bolotov 2006), a similar pattern to that found here has been reported for European grayling (*Thymallus thymallus*; Koskinen *et al.* 2000). The deep divergence and largely non-overlapping distributions of the two lineages suggest distinct postglacial colonization histories and

two postglacial dispersal routes into Fennoscandia. The analyses of nuclear loci supported the inference based on mtDNA data, but the resolution they provided was low, perhaps due to strong bottleneck effects (Takezaki & Nei 1996; see also below). A notable finding in our mtDNA analyses was that the genetic diversity in the eastern lineage was much lower as compared to the western lineage. The fact that one haplotype (E3) was widely distributed throughout the eastern lineage suggests that the individuals of the eastern lineage share a common ancestry. Nevertheless, given the sparse sampling in the western Fennoscandia, further sampling and studies are required to assess levels of genetic diversity in the western lineage.

Genetic structure and colonization pattern

The analyses of nuclear loci provided further insights into the population history and structure of Fennoscandian nine-spined sticklebacks. The most interesting finding was that genetic variation in the eastern lineage decreased dramatically and systematically with increasing latitude independently of habitat type. The pattern is in agreement with the theoretical expectation that northward expansion from southern refugia disposes populations to serial founder effects leading to gradual loss of genetic variation towards the north (Hewitt 1996; Austerlitz *et al.* 1997). Northward reduction in genetic variation has been also demonstrated in some other – mainly terrestrial – organisms in Fennoscandia (Merilä *et al.* 1996, 1997; Palo *et al.* 2004; Johansson *et al.* 2006; Tollefsrud *et al.* 2009) and elsewhere (Schmitt & Seitz 2002; Gysels *et al.* 2004; Adams *et al.* 2006; Rowe & Beebee 2007). Despite their low genetic variability, no consistent and significant signatures of transient population bottlenecks were identified neither in the case of (small) pond sites, nor in the case of mid- and northern Fennoscandia sites. In fact, according to our own observations, nine-spined sticklebacks are abundant and occur in high densities in these sites. The interpretation that the northward reduction of genetic variability is caused by historical rather than contemporary factors was further supported by the fact that the estimates of historical effective population sizes decreased – parallel to within population genetic variability – with increasing latitude, independent of habitat type. Hence, the observed patterns of latitudinally ordered genetic diversity are difficult to understand other than in the light of sequential reduction of genetic variability during the recolonization process that has taken place from south to north.

Despite a recent origin of Fennoscandian nine-spined sticklebacks, the degree of genetic differentiation within the eastern lineage was very high and estimated F_{ST} values (freshwater average F_{ST} = 0.49; maximum F_{ST} = 0.97) are among the highest reported for any fish species so far (e.g. Hänfling *et al.* 2002; Jacobsen *et al.* 2005; Vonlanthen *et al.* 2007; Barson *et al.* 2009). In particular, strong population subdivision was found in mid- and northern Fennoscandian freshwater sites, as expected in bottlenecked populations due to founder effects (Chakraborty & Nei 1977; Le & Kremer 1998; Hedrick 1999). In line with this trend, a Bayesian clustering analysis assigned the individuals of mid- and northern Fennoscandia to several different clusters, while most individuals from southern Fennoscandia were included in a single cluster. These align with the observed lack of isolation by distance in eastern lineage to support the resolution that these populations have been subject to strong genetic drift in the past (Hutchison & Templeton 1999; Koizumi *et al.* 2006). Hence, the facts reviewed above suggest that the nine-spined sticklebacks of the eastern lineage have colonized Fennoscandia from the south and have become genetically fragmented due to founder events associated with this colonization process.

The levels of genetic variability in all sites belonging to the western lineage were relatively high and comparable to that in the most southern Fennoscandian sites belonging to the eastern lineage. No geographic trends in levels of genetic variability were found in western lineage, but data was limited both in terms of number of sites and their coverage. Hence, more detailed inference about history and variability of the western lineage should await denser sampling and samples from southern Sweden and mid-Norway would be particularly interesting for investigations to come: especially in the view that the estimated historical effective population sizes were relatively large for the French and Belgian sites, which were non-glaciated during the Pleistocene glaciations. Hence, they may represent refugial areas of the western lineage.

Differentiation in coastal vs. freshwater habitats

The degree of genetic differentiation among freshwater sites exceeded that among coastal sites, conforming to the typical pattern among fishes (Gyllensten 1985; Ward *et al.* 1994; DeWoody & Avise 2000). Higher rates of gene flow or larger effective population sizes in marine environments, are possible explanations for this difference. In fact, historical migration rates were suggested to be higher among the coastal than among the freshwater sites, but historical effective population sizes were suggested to be similar in both habitat types. Interestingly, the degree of genetic differentiation between the Baltic and the White Sea sites was relatively low (F_{ST} = 0.122), but still much higher than

that among the Baltic Sea sites (F_{ST} = 0.011). The low degree of genetic differentiation between the Baltic and the White Sea sites in both nuclear and mtDNA data suggests that the divergence is of postglacial origin, rather than due to colonization from different refugial areas.

In contrast to coastal sites, freshwater sites are confined to physically finite habitats. As such, we expected to observe reduced intrapopulation diversity and increased interpopulation differentiation in ponds as compared to lakes and rivers. However, no clear-cut habitat dependent patterns were discovered neither in genetic variability or degree of genetic differentiation, nor in historical effective population sizes. While we acknowledge the small number of replicate sites within different categories of freshwater habitats, it appears that historical events, rather than contemporary habitat type effects, have been driving the patterns of genetic population structure in our data. This is in contrast with results of some recent studies where the impacts of contemporary eco-demographic factors were suggested to override the historical effects on genetic variability (e.g. Johansson *et al.* 2006).

Nine-spined vs. three-spined sticklebacks

In most parts of their European distribution, nine-spined sticklebacks occur in sympatry with three-spined sticklebacks (Paepke 2001). Both species inhabit coastal waters, but the distribution of the nine-spined sticklebacks extends further inland than that of the three-spined stickleback (Paeple 2001). In contrast to nine-spined sticklebacks, a single ancestral marine lineage of three-spined sticklebacks appears to have colonized the freshwater habitats of Fennoscandia (Mäkinen *et al.* 2006; Mäkinen & Merilä 2008). Our results suggest that in the case of nine-spined sticklebacks, Fennoscandia has been colonized by at least two different lineages of nine-spined sticklebacks, possibly of freshwater origin. Indirect – and yet largely hypothetical – support for the freshwater origin is provided by their widespread occurrence in inland areas, as well by their genetic characteristics.

In a review of microsatellite polymorphisms in fish, DeWoody & Avise (2000) reported that an average heterozygosity for marine, anadromous and freshwater fish was 0.77, 0.68 and 0.54, respectively. In the Baltic and White Sea sites of three-spined sticklebacks, heterozygosity was high (H_E = 0.80; Mäkinen *et al.* 2006) as is typical for marine fish species. However, heterozygosity in the Baltic and White Sea sites of nine-spined sticklebacks is lower (H_E = 0.58), and appears to correspond more closely to that of freshwater rather than marine fish species. Hence, the relatively low genetic variation in the coastal nine-spined sticklebacks might be due to their freshwater origin. Alternatively, the low genetic variation in nine-spined sticklebacks might reflect ascertainment bias since the loci used in this study were originally developed for three-spined sticklebacks (Mäkinen *et al.* 2007). Hence, while the question about the freshwater vs. marine origin of the nine-spined sticklebacks cannot yet be conclusively resolved, it is nevertheless clear that the differences in present-day levels and distribution of genetic variability between three-spined and nine-spined sticklebacks owe to differences in their colonization history of the northern Europe.

Conclusions

In summary, our study demonstrates how postglacial colonization can influence the contemporary population genetic structure of a species, indicating a strong historical component to the patterns of genetic variation and differentiation in northern European nine-spined sticklebacks. As expected, the levels of genetic variation and differentiation were significantly different between the coastal and freshwater sites. However, the genetic structure of the freshwater sites was not much influenced by habitat type effects, but strong negative association between latitude and intrapopulation genetic variability was observed. Historical northward expansions of this species were most likely the cause of low genetic variation and strong genetic subdivision in mid and northern Fennoscandia. The geographically structured pattern of genetic diversity suggests that the contemporary genetic structure of freshwater nine-spined sticklebacks has been strongly impacted by the founder events associated with postglacial recolonization of the northern Europe.

Acknowledgements

We thank Abigel Gonda, Kjartan Østbye, Tom Olav Klepaker, Jörg Freyhof, Tuomas Leinonen, Jaakko Lumme, Hannu Mäkinen, Kimmo Kahilainen, Henri Persat, Anti Vasemägi, Akira Goto, Tom Pike, Arne Levsen, Joost Raeymaekers, Taina Kojola, Leena Eerola, Aki Hirvonen, Lennart Persson, Jarmo Saarikivi, Pirkko Siikamäki, Victor Berger, Göran Englund, Daniel Lussetti and Kalevi Kuusela for help in obtaining samples. Special thanks to the Oulanka Research Station and the White Sea Biological Station in helping us with sampling some remote areas. Thanks are also due to Hannu Mäkinen for his comments and Jacquelin DeFaveri for correcting the English. Our study was supported by the Academy of Finland, the Baltgene-project of the BONUS-program and the Japan Society for the Promotion of Science.

References

Adams SM, Lindmeier JB, Duvernell DD (2006) Microsatellite analysis of the phylogeography, Pleistocene history and

secondary contact hypotheses for the killifish, *Fundulus heteroclitus*. *Molecular Ecology*, **15**, 1109–1123.
Austerlitz F, Jung-Muller B, Godelle B, Gouyon P (1997) Evolution of coalescence times, genetic diversity and structure during colonization. *Theoretical Population Biology*, **51**, 148–164.
Barson NJ, Cable J, Van Oosterhout C (2009) Population genetic analysis of microsatellite variation of guppies (*Poecilia reticulata*) in Trinidad and Tobago: evidence for a dynamic source-sink metapopulation structure, founder events and population bottlenecks. *Journal of Evolutionary Biology*, **22**, 485–497.
Beerli P (2004) Effect of unsampled populations on the estimation of population sizes and migration rates between sampled populations. *Molecular Ecology*, **13**, 827–836.
Beerli P, Felsenstein J (2001) Maximum likelihood estimation of a migration matrix and effective population sizes in n subpopulations by using a coalescent approach. *Proceedings of the National Academy of Sciences, USA*, **98**, 4563–4568.
Bell MA, Foster SA (1994) Introduction to the evolutionary biology of the threespine stickleback. In: *The Evolutionary Biology of the Threespine Stickleback* (eds Bell MA, Foster SA), pp. 1–27. Oxford University Press, Oxford.
Bernatchez L, Wilson C (1998) Comparative phylogeography of Nearctic and Palearctic fishes. *Molecular Ecology*, **7**, 431–452.
Brownstein MJ, Carpten JD, Smith JR (1996) Modulation of non-templated nucleotide addition by *Taq* DNA polymerase: primer modifications that facilitate genotyping. *BioTechniques*, **20**, 1004–1010.
Chakraborty R, Nei M (1977) Bottleneck effects on average heterozygosity and genetic distance with the stepwise mutation model. *Evolution*, **31**, 347–356.
Colosimo PF, Hosemann KE, Balabhadra S *et al.* (2005) Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. *Science*, **307**, 1928–1933.
Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics*, **144**, 2001–2014.
DeWoody JA, Avise JC (2000) Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. *Journal of Fish Biology*, **56**, 461–473.
Di Rienzo A, Peterson AC, Garza JC *et al.* (1994) Mutational processes of simple-sequence repeat loci in human populations. *Proceedings of the National Academy of Sciences, USA*, **91**, 3166–3170.
Eckert CG, Samis KE, Lougheed SC (2008) Genetic variation across species' geographical ranges: the central-marginal hypothesis and beyond. *Molecular Ecology*, **17**, 1170–1188.
Elphinstone MS, Hinten GN, Anderson MJ, Nock CJ (2003) An inexpensive and high-throughput procedure to extract and purify total genomic DNA for population studies. *Molecular Ecology Notes*, **3**, 317–320.
Estoup A, Angers B (1998) Microsatellites and minisatellites for molecular ecology: theoretical and empirical considerations. In: *Advances in Molecular Ecology* (ed. Carvlho GR), pp. 55–86. NATO Science Series, IOS Press, Amsterdam.
Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology*, **14**, 2611–2620.
Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes—application to human mitochondrial-DNA restriction data. *Genetics*, **131**, 479–491.
Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47–50.
Goudet J (2001) *FSTAT: A Computer Program to Estimate and Test Gene Diversities and Fixation Indices* (version 2.9.3). Available at http://www2.unil.ch/popgen/softwares/fstat.htm.
Gyllensten U (1985) The genetic structure of fish: differences in the intraspecific distribution of biochemical genetic variation between marine, anadromous, and freshwater species. *Journal of Fish Biology*, **26**, 691–699.
Gysels ES, Hellemans B, Pampoulie C, Volckaert FAM (2004) Phylogeography of the common goby, *Pomatoschistus microps*, with particular emphasis on the colonization of the Mediterranean and the North Sea. *Molecular Ecology*, **13**, 403–417.
Haglund TR, Buth DG, Lawson R (1992) Allozyme variation and phylogenetic relationships of Asian, North American, and European populations of the ninespine stickleback, *Pungitius pungitius*. In: *Systematics, Historical Ecology, and North American Freshwater Fishes* (ed. Mayden RL), pp. 438–452. Stanford University Press, Stanford.
Hänfling B, Hellemans B, Volckaert FA, Carvalho GR (2002) Late glacial history of the cold-adapted freshwater fish *Cottus gobio*, revealed by microsatellites. *Molecular Ecology*, **11**, 1717–1729.
Hedrick PW (1999) Perspective: highly variable loci and their interpretation in evolution and conservation. *Evolution*, **53**, 313–318.
Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society*, **58**, 247–276.
Hewitt GM (1999) Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society*, **68**, 87–112.
Hewitt G (2000) The genetic legacy of the Quaternary ice ages. *Nature*, **405**, 907–913.
Hewitt GM (2004) The structure of biodiversity—insights from molecular phylogeography. *Frontiers in Zoology*, **1**, 4.
Hutchison DW, Templeton AR (1999) Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. *Evolution*, **53**, 1898–1914.
Jacobsen B, Hansen MM, Loeschcke V (2005) Microsatellite DNA analysis of northern pike (*Esox lucius* L.) populations: insights into the genetic structure and demographic history of a genetically depauperate species. *Biological Journal of the Linnean Society*, **84**, 91–101.
Johansson M, Primmer CR, Merilä J (2006) History vs. current demography: explaining the genetic population structure of the common frog (*Rana temporaria*). *Molecular Ecology*, **15**, 975–983.
Kalinowski ST (2005) HP-RARE 1.0: A computer program for performing rarefaction on measures of allelic richness. *Molecular Ecology Notes*, **5**, 187–189.
Kocher TD, Thomas WK, Meyer A *et al.* (1989) Dynamics of mitochondrial DNA evolution in mammals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences, USA*, **86**, 6196–6200.
Koizumi I, Yamamoto S, Maekawa K (2006) Decomposed pairwise regression analysis of genetic and geographic distances reveals a metapopulation structure of stream-dwelling Dolly Varden charr. *Molecular Ecology*, **15**, 3175–3189.
Koskinen MT, Ranta E, Piironen J *et al.* (2000) Genetic lineages and postglacial colonization of grayling (*Thymallus thymallus*, Salmonidae) in Europe, as revealed by mitochondrial DNA analyses. *Molecular Ecology*, **9**, 1609–1624.
Langella O (2002) Populations 1.2.28. Logiciel de génétique des populations. Laboratoire Populations, génétique et évolution, CNRS UPR9034, Gif-sur-Yvette.
Largiadèr CR, Fries V, Kobler B, Bakker TC (1999) Isolation and characterization of microsatellite loci from the three-spined stickleback (*Gasterosteus aculeatus* L.). *Molecular Ecology*, **8**, 342–344.
Le CorreV, Kremer A (1998) Cumulative effects of founding events during colonisation on genetic diversity and differentiation in an island and stepping-stone model. *Journal of Evolutionary Biology*, **11**, 495–512.
Lippe C, Dumont P, Bernatchez L (2006) High genetic diversity and no inbreeding in the endangered copper redhorse, *Moxostoma hubbsi* (Catostomidae, Pisces): the positive sides of a long generation time. *Molecular Ecology*, **15**, 1769–1780.
Makhrov AA, Bolotov IN (2006) Dispersal routes and species identification of freshwater animals in Northern Europe: a review of molecular evidence. *Russian Journal of Genetics*, **42**, 1101–1115.
Mäkinen HS, Merilä J (2008) Mitochondrial DNA phylogeography of the three-spined stickleback (*Gasterosteus aculeatus*) in Europe—evidence for multiple glacial refugia. *Molecular Phylogenetics and Evolution*, **46**, 167–182.
Mäkinen HS, Cano JM, Merilä J (2006) Genetic relationships among marine and freshwater populations of the European three-spined stickleback (*Gasterosteus aculeatus*) revealed by microsatellites. *Molecular Ecology*, **15**, 1519–1534.
Mäkinen HS, Välimäki K, Merilä J (2007) Cross-species amplification of microsatellite loci for nine-spined stickleback *Pungitius pungitius*. *Annales Zoologici Fennici*, **44**, 218–224.
Mattern MY (2004) Molecular phylogeny of the Gasterosteidae: the importance of using multiple genes. *Molecular Phylogenetics and Evolution*, **30**, 366–377.
McPhail JD (1963) Geographic variation in North American ninespine sticklebacks, *Pungitius pungitius*. *Journal of the Fisheries Research Board of Canada*, **20**, 27–44.
Merilä J, Björklund M, Baker AJ (1996) Genetic population structure and gradual northward decline of genetic variability in the greenfinch (*Carduelis chloris*). *Evolution*, **50**, 2548–2557.
Merilä J, Björklund M, Baker AJ (1997) Historical demography and present day population structure of the greenfinch, *Carduelis chloris*—an analysis of mtDNA control-region sequences. *Evolution*, **51**, 946–956.
Muller MH, Leppälä J, Savolainen O (2008) Genome-wide effects of postglacial colonization in *Arabidopsis lyrata*. *Heredity*, **100**, 47–58.
Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.
Nei M, Tajima F, Tateno Y (1983) Accuracy of estimated phylogenetic trees from molecular data. *Journal of Molecular Evolution*, **19**, 153–170.

Nylander JAA (2004) *MrModeltest v2. Program distributed by the author*. Evolutionary Biology Centre, Uppsala University.
Paepke HJ (2001) *Pungitius pungitius* (Linnaeus, 1758). In: *The Freshwater Fishes of Europe* (eds Bănărescu P, Paepke HJ), Vol. 5, pp. 277–299. Aula-Verlag, Wiebelsheim.
Palo JU, Schmeller D, Laurila A *et al.* (2004) High degree of population subdivision in a widespread amphibian. *Molecular Ecology*, **13**, 2631–2644.
Palumbi SR (1996) Nucleic acids. II. The polymerase chain reaction. In: *Molecular Systematics*, 2nd edn (eds Hillis DM, Moritz C, Mable BK), pp. 205–248. Sinauer Associates, Sunderland, MA.
Pamilo P, Savolainen O (1999) Post-glacial colonization, drift, local selection and conservation value of populations: a northern perspective. *Hereditas*, **130**, 229–238.
Peichel CL, Nereng KS, Ohgi KA *et al.* (2001) The genetic architecture of divergence between threespine stickleback species. *Nature*, **414**, 901–905.
Piry S, Luikart G, Cornuet JM (1999) BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity*, **90**, 502–503.
Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.
Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.
Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.
Rousset F (2008) Genepop '007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Notes*, **8**, 103–106.
Rowe G, Beebee TJC (2007) Defining population boundaries: use of three Bayesian approaches with microsatellite data from British natterjack toads (*Bufo calamita*). *Molecular Ecology*, **16**, 785–796.
Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, **19**, 2496–2497.
Sagarin RD, Gaines SD, Gaylord B (2006) Moving beyond assumptions to understand abundance distributions across the ranges of species. *Trends in Ecology & Evolution*, **21**, 524–530.
Schmitt T (2007) Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. *Frontiers in Zoology*, **4**, 11.
Schmitt T, Seitz A (2002) Postglacial distribution area expansion of *Polyommatus coridon* (Lepidoptera: Lycaenidae) from its Ponto-Mediterranean glacial refugium. *Heredity*, **89**, 20–26.
Swofford DL (2002) *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods)*, version 4.0b10. Sinauer Associates, Sunderland, MA.
Taggart JB, Hynes RA, Prodöhl PA, Ferguson A (1992) A simplified protocol for routine total DNA isolation from salmonid fishes. *Journal of Fish Biology*, **40**, 963–965.
Takata K, Goto A, Yamazaki F (1987) Biochemical identification of a brackish water type of *Pungitius pungitius*, and its morphological and ecological features in Hokkaido, Japan. *Japanese Journal of Ichthyology*, **34**, 176–183.

Takezaki M, Nei M (1996) Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics*, **144**, 389–399.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24**, 1596–1599.

Tollefsrud MM, Sønstebø JH, Brochmann C *et al*. (2009) Combined analysis of nuclear and mitochondrial markers provide new insight into the genetic structure of North European *Picea abies*. *Heredity*, **102**, 549–562.

Tomiuk J, Guldbrandtsen B, Loeschcke V (1998) Population differentiation through mutation and drift—a comparison of genetic identity measures. *Genetica*, **102/103**, 545–558.

Tonteri A, Veselov AJe, Titov S *et al*. (2007) The effect of migratory behaviour on genetic diversity and population divergence: a comparison of anadromous and freshwater Atlantic salmon *Salmo salar*. *Journal of Fish Biology*, **70** (Suppl. C), 381–398.

Van Oosterhout C, Hutchinson WF, Wills DP, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Vonlanthen P, Excoffier L, Bittner D *et al*. (2007) Genetic analysis of potential postglacial watershed crossings in Central Europe by the bullhead (*Cottus gobio* L.). *Molecular Ecology*, **16**, 4572–4584.

Vucetich JA, Waite TA (2003) Spatial patterns of demography and genetic processes across the species' range: null hypotheses for landscape conservation genetics. *Conservation Genetics*, **4**, 639–645.

Ward RD, Woodwark M, Skibinski DOF (1994) A comparison of genetic diversity levels in marine, freshwater, and anadromous fishes. *Journal of Fish Biology*, **44**, 213–232.

Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

Wootton RJ (1976) *The Biology of the Sticklebacks*. Academic Press, New York.

T.S. and Y.S. are broadly interested in fish population genetics and genomics, and those in three- and nine-spined sticklebacks in particular. G.H.'s interests centre around evolutionary studies of nine-spined sticklebacks. J.M. is interested in evolutionary and population genetics of wild vertebrate populations with increasing curiosity directed towards the study of sticklebacks.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Multidimensional scaling plot of pairwise D_A distances (A) and distribution of PC1 and PC2 scores in principal component analysis of the allele frequencies (B) in nine-spined sticklebacks. Inverted triangle: coastal, circle: lake, square: river, triangle: pond. Closed symbols: eastern lineage, open symbols: western lineage, x: Japanese sample.

Table S1 Sampling sites, sample sizes (n), number of observed alleles (A), allelic richness (Ar), expected heterozygosity (H_E) and F_{IS} in nine-spined sticklebacks

Table S2 Variable nucleotide sites across 1104 bp sequences of cytochrome *b* in nine-spined sticklebacks. Complete sequences have been deposited in GenBank under accession numbers GU227740–GU227783.

Table S3 Estimates of migration parameter M within freshwater or coastal sites and between them in nine-spined sticklebacks

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Genetic Architecture of Parallel Pelvic Reduction in Ninespine Sticklebacks

Takahito Shikano,*,[2] Veronika N. Laine,[†] Gábor Herczeg,*,[1] Johanna Vilkki,[‡] and Juha Merilä*
*Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, FI-00014, Helsinki, Finland, [†]Division of Genetics and Physiology, Department of Biology, University of Turku, FI-20014, Turku, Finland, and [‡]MTT Agrifood Research Finland, FI-31600, Jokioinen, Finland

ABSTRACT Teleost fish genomes are known to be evolving faster than those of other vertebrate taxa. Thus, fish are suited to address the extent to which the same *vs.* different genes are responsible for similar phenotypic changes in rapidly evolving genomes of evolutionary independent lineages. To gain insights into the genetic basis and evolutionary processes behind parallel phenotypic changes within and between species, we identified the genomic regions involved in pelvic reduction in Northern European ninespine sticklebacks (*Pungitius pungitius*) and compared them to those of North American ninespine and threespine sticklebacks (*Gasterosteus aculeatus*). To this end, we conducted quantitative trait locus (QTL) mapping using 283 F_2 progeny from an interpopulation cross. Phenotypic analyses indicated that pelvic reduction is a recessive trait and is inherited in a simple Mendelian fashion. Significant QTL for pelvic spine and girdle lengths were identified in the region of the *Pituitary homeobox transcription factor 1* (*Pitx1*) gene, also responsible for pelvic reduction in threespine sticklebacks. The fact that no QTL was observed in the region identified in the mapping study of North American ninespine sticklebacks suggests that an alternative QTL for pelvic reduction has emerged in this species within the past 1.6 million years after the split between Northern European and North American populations. In general, our study provides empirical support for the view that alternative genetic mechanisms that lead to similar phenotypes can evolve over short evolutionary time scales.

KEYWORDS
chromosomal rearrangement
genetic convergence
linkage map
parallel evolution
QTL

Understanding the genetic basis of the evolution and diversity of phenotypic traits is a central topic in evolutionary biology. Similar phenotypes often evolve independently across multiple populations or closely related species facing similar environmental selection pressures (Barrett and Schluter 2008; Nadeau and Jiggins 2010). Such parallel phenotypic changes have been observed in a wide variety of organisms (Jones *et al.* 1992; Losos *et al.* 1998; Huey *et al.* 2000; Colosimo *et al.* 2005; Rogers and Bernatchez 2007), strongly suggesting that the observed phenotypic parallelism is shaped by natural selection (Endler 1986; Schluter 2000). Parallel phenotypic evolution can be based on utilization of the same or different genetic mechanisms to acquire the same phenotypes (Arendt and Reznick 2008; Elmer and Meyer 2011). Although several empirical studies have provided examples in which similar phenotypes are produced by the repeated selection of the same pool of standing variation (Barrett and Schluter 2008; Nadeau and Jiggins 2010), the relative importance of standing variation and new mutations in parallel phenotypic evolution is as yet poorly understood (Elmer and Meyer 2011; Conte *et al.* 2012).

Comparative genome mapping provides a framework to assess the similarities and dissimilarities in the genetic bases of phenotypic evolution both at intraspecific and interspecific levels, and also offers an opportunity to gain insights into the evolutionary history of genomes. Recent studies have discovered that the rate of genome evolution differs among taxonomic groups (Kohn *et al.* 2006; Ferguson-Smith and Trifonov 2007; Huttley *et al.* 2007; Hufton *et al.* 2008). For instance, teleost fish genomes exhibit a higher incidence of chromosomal rearrangements and a faster evolution of protein coding sequences than other vertebrate taxa, implying that their genomes are evolving rapidly (Jaillon *et al.* 2004; Naruse *et al.* 2004; Woods *et al.* 2005; Sémon and Wolfe 2007; Hufton *et al.* 2008). Thus, as seen in frequent turnover and rearrangements of fish sex chromosomes (Devlin and Nagahama 2002; Mank and Avise 2009; Cioffi *et al.* 2012; Kitano and Peichel 2012), fish genomes are considered plastic. The rapid evolution of fish genomes is associated with the high morphological, ecological, and physiological diversity in this taxon (Ravi and Venkatesh 2008), and parallel phenotypic evolution has been observed in several fish species (Bell and Foster 1994; Reznick *et al.* 1996; Landry *et al.* 2007; Jeffery 2009; Chan *et al.* 2010). Therefore, teleost fish are well-suited for studying the evolutionary dynamics and processes of genome rearrangements, as well as the genetic basis of parallel phenotypic evolution. In particular, they are useful to address the extent to which the same genes can be responsible for similar phenotypic changes in rapidly evolving genomes.

Stickleback fishes have served as important model organisms in evolutionary biology (Wootton 1976; Bell and Foster 1994; Kingsley and Peichel 2007; Östlund-Nilsson and Mayer 2007; Merilä 2013). In particular, threespine (*Gasterosteus aculeatus*) and ninespine sticklebacks (*Pungitius pungitius*) exhibit diverse phenotypic and ecological characteristics across their global distributions (Bell and Foster 1994; Merilä 2013). Although these species diverged from a common ancestor approximately 13 million years ago (Bell *et al.* 2009), they share similar morphological features, including bony lateral plates and pelvic apparatus. Thus, they are well-suited for studying the genetic basis of phenotypic evolution at both intraspecific and interspecific levels. Parallel phenotypic evolution has been widely observed and extensively studied in threespine sticklebacks (Bell 1976; Bell and Foster 1994; Walker and Bell 2000). Specifically, although ancestral marine threespine sticklebacks have a full row of lateral armor plates, long pelvic spines, and well-developed pelvic girdles, all of these traits have become reduced or even lost in independently and repeatedly colonized freshwater populations in which predation pressure by piscine predators is reduced. Genetic analyses have demonstrated that the repeated reduction in the number of lateral bony plates is governed by the *Ectodysplasin* (*Eda*) gene in globally distributed populations (Colosimo *et al.* 2004, 2005; Cresko *et al.* 2004). Similarly, the *Pituitary homeobox transcription factor 1* (*Pitx1*) gene has been shown to be responsible for pelvic reduction in both Pacific and Atlantic populations of threespine sticklebacks (Cole *et al.* 2003; Cresko *et al.* 2004; Shapiro *et al.* 2004; Coyle *et al.* 2007; Chan *et al.* 2010). Although fewer genetic studies have been conducted using ninespine sticklebacks, phenotypic analyses of F_1 hybrids between North American ninespine and threespine sticklebacks suggested that pelvic reduction is controlled by the same gene in these species (Shapiro *et al.* 2006). Nevertheless, a quantitative trait locus (QTL) mapping study of North American ninespine sticklebacks indicated that a major genomic region influencing pelvic reduction is distinct from the *Pitx1* locus (Shapiro *et al.* 2009). Thus, genetic variation for pelvic reduction is likely to exist at multiple loci in this species. Phylogenetic analyses have uncovered the presence of several distinct lineages in ninespine sticklebacks across the Northern Hemisphere (Haglund *et al.* 1992a; Takahashi and Goto 2001; Aldenhoven *et al.* 2010; Shikano *et al.* 2010a; Teacher *et al.* 2011). Although threespine sticklebacks have undergone rapid morphological transitions after freshwater colonization, mainly after the last ice age (Bell and Foster 1994), morphological diversification might have occurred through different genetic changes in distinct lineages—and in different time scales—in ninespine sticklebacks because of their more heterogeneous evolutionary history. Therefore, comparative analyses of these two stickleback species can provide important insight into the evolutionary processes and genetic underpinnings that have led to similar phenotypes.

The main aim of this study was to improve our understanding of the genetic basis of parallel pelvic reduction in ninespine sticklebacks and to ask whether this reduction is likely to have occurred through similar or different genetic mechanisms in different lineages. In particular, we were interested in exploring the origin and evolution of genetic variation for pelvic reduction in this species. To this end, we identified the genomic regions involved in pelvic reduction in Northern European ninespine sticklebacks—with the aid of QTL mapping with 283 F_2 segregating progeny of the cross between populations with and without pelvic spines—and compared them to those of North American ninespine and threespine sticklebacks (Cole *et al.* 2003; Cresko *et al.* 2004; Shapiro *et al.* 2004, 2006, 2009; Coyle *et al.* 2007; Chan *et al.* 2010). In addition, intraspecific and interspecific comparative genomic analyses were conducted to assess possible chromosomal rearrangements by comparing our linkage map with that of North American ninespine sticklebacks (Shapiro *et al.* 2009) and the threespine stickleback genome (Jones *et al.* 2012). Given that the stickleback sex chromosomes are known to evolve in rapid pace (Ross *et al.* 2009), we also mapped the sex-determining locus in the European lineage of ninespine sticklebacks to see whether it corresponds to that found in the North American lineage of this species.

MATERIALS AND METHODS

Fish

The grandparental fish (F_0) were collected from the Baltic Sea (Helsinki, Finland; 60°13′N, 25°11′E) and a pond (Rytilampi, Finland; 66°23′N, 29°19′E) in northeastern Finland in 2006. Pelvic reduction was observed in the pond population, whereas no pelvic reduction was found in the marine population (Herczeg *et al.* 2010). A female from the marine population was artificially crossed with a male from the pond population in July 2006, and the F_1 offspring were reared in an aquarium at approximately 15°. They were fed brine shrimp (*Artemia* sp.) nauplii in larval and juvenile stages, and, later, frozen bloodworms (*Chironomidae* sp.). After an artificial hibernation at 6° without light, fish were maintained at 17° under permanent light to facilitate reproduction. One female and one male were randomly chosen and mated in an aquarium. Seven successive clutches were obtained in September and October 2008. These clutches were reared in 1.4-liter tanks in a zebrafish rack system equipped with physical, biological, and UV filters (Aquaneering Inc., San Diego, CA). The F_2 offspring were placed individually in 1.4-liter tanks in the zebrafish rack systems 6 d after hatching. White plastic sheets were placed between the tanks to block visibility between the fish. The fish were reared at 17° under the 14-hr light and 10-hr dark photoperiod and fed twice per day. Food was, at first, brine shrimp nauplii. After 4 wk, it was changed to brine shrimp nauplii and frozen *Cyclops*. After another 4 wk, it was changed to frozen *Cyclops* and bloodworms. Finally, after another 4 wk, it was changed to frozen bloodworms. At 187 days posthatching, the fish were anesthetized with MS-222 (tricaine methanesulfonate) and photographed with a digital camera. The specimens were fixed in 4% formalin for morphological measurements. Fin clips were preserved in ethanol for DNA analyses. In total, 283 fish (56, 44, 38, 37, 40, 31, and 27 from seven clutches) were used for analyses. The experiments were conducted under the license from the Finnish National Animal Experiment Board (#STH379A).

doi: 10.1534/g3.113.007237
Manuscript received June 25, 2013; accepted for publication August 17, 2013

Supporting information is available online at http://www.g3journal.org/lookup/suppl/doi:10.1534/g3.113.007237/-/DC1
[1]Present address: Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/C, 1117, Hungary.
[2]Corresponding author: Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, P.O. Box 65, FI-00014, Helsinki, Finland. E-mail: takahito.shikano@helsinki.fi

Morphological measurements

The fish were stained with Alizarin Red S, following the study of Pritchard and Schluter (2001). Pelvic spine and girdle lengths were measured with a digital caliper to the nearest 0.01 mm. Both left and right pelvic girdles and spines were measured twice by the same person, and the averaged values were used for analyses. As a size proxy, we calculated centroid size, which is the square root of the summed squared distances from the landmarks to the centroid (Bookstein 1991). We used the same landmarks on the digital photographs as outlined by Herczeg *et al.* (2010), and we calculated centroid size using tpsRelw 1.46 (Rohlf 2006). Gender was determined by checking the gonads.

DNA extraction and genotyping

Total genomic DNA was extracted from ethanol-preserved fin clips using a silica fine-based purification method (Elphinstone *et al.* 2003) after proteinase K digestion. All the F_2 offspring (N = 283) and their parents and grandparents (N = 4) were genotyped for 235 microsatellite markers (Largiadèr *et al.* 1999; Peichel *et al.* 2001; Heckel *et al.* 2002; Colosimo *et al.* 2004; Miller *et al.* 2007; Mäkinen *et al.* 2008; Shapiro *et al.* 2009; Shikano *et al.* 2010b, 2011; Shimada *et al.* 2011; Laine *et al.* 2012; Supporting Information, Table S1). Out of the 235 markers, 46 were developed for specific genes with known biological functions in fish (Shapiro *et al.* 2009; Shikano *et al.* 2010b; Shimada *et al.* 2011; Laine *et al.* 2012). Polymerase chain reactions (PCRs) for all markers except Ppbig were performed in a 10-μl volume containing 1× Qiagen Multiplex PCR Master Mix (Qiagen), 0.5× Q-Solution, 2 pmol of each primer, and 10–20 ng of template DNA. One of each primer pair was labeled with FAM, HEX, or TET fluorescent dye. PCR cycling started with an initial activation step at 95° for 15 min, followed by 30 cycles of 94° for 30 sec, 55° for 90 sec, and 72° for 60 sec, and completed with a final extension at 60° for 5 min. PCRs for Ppbig markers were conducted according to the methods of Laine *et al.* (2012). All PCR products were diluted 1:500 with Milli-Q water and genotyped using a MegaBACE 1000 automated sequencer (Amersham Biosciences) with ET-ROX 400 size standard (Amersham Biosciences). Alleles were scored using Fragment Profiler 1.2 program (Amersham Biosciences) and edited by eye. To ensure consistency in genotyping, all alleles were read by the same person.

Linkage map

A linkage map was constructed using improved CRI-MAP 2.5 (Green *et al.* 1990). The logarithm of the odds (LOD) scores for all pairs of markers were obtained using the TWOPOINT option. LOD score threshold of 3.0 was used as a significant criterion for linkage. The linkage map of North American ninespine sticklebacks (Shapiro *et al.* 2009) and the threespine stickleback genome sequence (Ensembl, database v. 66.1) were used as a reference for the initial linkage group (LG) building. For each LG, the best order of the markers was determined using the BUILD option by beginning with the most informative marker pair. Markers that could not be fitted straight with BUILD and with LOD score ≥4.0 were fitted manually. The FLIPS option (N = 3–5) was used to evaluate the statistical significance of the obtained order. After the best order was determined within each LG, double recombination events were detected using the CHROMPIC option. Individuals with more than four recombinations were removed and a second CRIMAP analysis round was conducted. Out of the 235 markers used for linkage analyses, nine with low polymorphism were discarded because of low LOD scores in TWOPOINT. Linkage maps were drawn using MAPCHART 2.2 (Voorrips 2002).

Genomic synteny was investigated by comparing our linkage map with the threespine stickleback genome (Jones *et al.* 2012) and the linkage map of North American ninespine sticklebacks (Shapiro *et al.* 2009). For the markers developed specifically for ninespine sticklebacks, microsatellite flanking sequences were subject to BLASTN searches against the threespine stickleback genome to identify homologous genomic regions. BLAST hits were considered significant at a threshold of $E < 10^{-5}$. Out of 226 informative markers in our linkage map, 110 were used for the linkage map of North American ninespine sticklebacks (Shapiro *et al.* 2009).

QTL mapping

QTL analyses were conducted with 226 markers, each of which had genotyping success of the F_2 offspring between 74% and 99%. Sex-averaged linkage map distances were used for QTL mapping. The analyses were performed with GridQTL (Seaton *et al.* 2006) by using the BCF2 portlet and fitting both additive and dominance effects at 1-cM intervals (File S1, File S2, and File S3). Sex was included in the models as a fixed effect, and centroid size was included as a covariate. Experiment-wide and chromosome-wide significance levels of QTL were determined based on 10,000 permutations. QTL was considered significant when the F-value was more than the 5% experiment-wide threshold, and was considered suggestive when the F-value was more than the 5% chromosome-wide threshold. Confidence intervals were estimated with 10,000 bootstrap iterations. The proportion of the phenotypic variance explained by the QTL was calculated according to the methods of Zhou *et al.* (2006).

RESULTS

Linkage and comparative mapping

The genetic linkage map constructed with 226 informative markers consisted of 21 LGs (Figure S1), in accord with chromosome number based on cytogenetic analysis (Ocalewicz *et al.* 2008). The sex-averaged linkage map spanned 1,632.7 cM, with an average intermarker distance of 7.2 cM. The number of loci per LG ranged from 5 to 21, and the size of the LGs spanned from 31.1 to 121.3 cM (Table S2). The female map covered 2178.7 cM and the male map covered 1211.6 cM (Table S2). Out of the 226 markers mapped, 217 had significant similarity to sequences of the threespine stickleback genome (Table S1). All of the 217 markers except one were assigned to specific LGs of the threespine stickleback genome.

In the linkage map, 206 markers were located in LGs in accordance with those of the threespine stickleback genome (Table 1). Likewise, in a comparison with the linkage map of North American ninespine sticklebacks (Shapiro *et al.* 2009), 105 out of the 110 markers used in both studies were mapped to the same LGs (Table 1). Although 12 markers were located on LG7 in the threespine stickleback genome, six of them were mapped to LG12, together with a set of 15 markers belonging to LG12 in the threespine stickleback genome (Figure 1). In the 21 markers mapped to LG12, pairwise LOD scores between the markers belonging to threespine stickleback LG7 and LG12 were significant (≥3.0) in 30 out of 90 combinations. However, the six markers belonging to threespine stickleback LG7 showed no significant LOD scores in any of 48 pairwise comparisons with the markers mapped to LG7 (Table S3). In addition to the interchromosomal discordance, the marker order within each LG was consistently inverted between the threespine stickleback genome and the linkage map of Northern European ninespine sticklebacks in partial regions of several chromosomes, including LG1, LG4, LG5, LG8, LG9, LG11, and LG13 (Figure S2).

Table 1 Synteny between the Northern European ninespine stickleback and threespine stickleback genome and between the Northern European and North American ninespine sticklebacks

Species	Northern European Ninespine Stickleback																				
LG	LG1	LG2	LG3	LG4	LG5	LG6	LG7	LG8	LG9	LG10	LG11	LG12	LG13	LG14	LG15	LG16	LG17	LG18	LG19	LG20	LG21
Threespine stickleback																					
LG1	15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG2	—	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG3	—	—	9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG4	—	—	—	16	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—
LG5	—	—	1	—	9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG6	—	—	—	—	—	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG7	—	—	—	—	—	—	6	—	—	—	—	6	—	—	—	—	—	—	—	—	—
LG8	—	—	—	—	—	—	—	9	—	—	—	—	—	—	—	—	—	—	—	—	—
LG9	—	—	—	—	—	1	—	—	10	—	—	—	—	—	—	—	—	—	—	—	—
LG10	—	—	—	—	—	—	—	—	—	7	—	—	—	—	—	—	—	—	—	—	—
LG11	—	—	—	—	—	—	—	—	—	—	18	—	—	—	—	—	—	—	—	—	—
LG12	—	—	—	—	—	—	—	—	—	—	—	15	—	—	—	—	—	—	—	—	—
LG13	—	—	—	—	—	—	—	—	—	—	—	—	16	—	—	—	—	—	1	—	—
LG14	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—
LG15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	10	—	—	—	—	—	—
LG16	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	11	—	—	—	—	—
LG17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	7	—	—	—	—
LG18	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	6	—	—	—
LG19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	11	—	—
LG20	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	6	—
LG21	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	7
Unknown	2	—	—	—	—	—	2	—	1	—	—	—	1	1	—	—	2	1	—	—	—
North American ninespine stickleback																					
LG1A	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG1B	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG2	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG3	—	—	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG4	—	—	—	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG5A	—	—	—	—	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG5B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG6A	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG6B	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG7A	—	—	—	—	—	—	—	—	—	—	—	5	—	—	—	—	—	—	—	—	—
LG7B	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG8	—	—	—	—	—	—	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—
LG9A	—	—	—	—	—	—	—	—	3	—	—	—	—	—	—	—	—	—	—	—	—
LG9B	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—
LG10	—	—	—	—	—	—	—	—	—	5	—	—	—	—	—	—	—	—	—	—	—
LG11A	—	—	—	—	—	—	—	—	—	—	7	—	—	—	—	—	—	—	—	—	—
LG11B	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—
LG12	—	—	—	—	—	—	—	—	—	—	—	6	—	—	—	—	—	—	—	—	—
LG13	—	—	—	—	—	—	—	—	—	—	—	—	11	—	—	—	—	—	—	—	—
LG14A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG14B	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—
LG15A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—
LG15B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	—	—	—	—	—	—
LG16	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	6	—	—	—	—	—
LG17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	—	—	—	—
LG18	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—
LG19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	8	—	—
LG20A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—
LG20B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LG21	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3

The numbers of markers on respective linkage groups are indicated. The data of the North American ninespine stickleback are based on the work of Shapiro *et al.* (2009).

Mapping of the sex-determining locus

The sex-determining locus was mapped to LG12, with the peak value at 53.2 cM (95% C.I. = 52.0–55.0) in the male map (F = 159.1; LOD = 46.1; Figure 1). In this LG, the sex-specific maps of females and males were 233.7 cM and 56.8 cM, respectively, including six markers belonging to threespine stickleback LG7 (Figure 1).

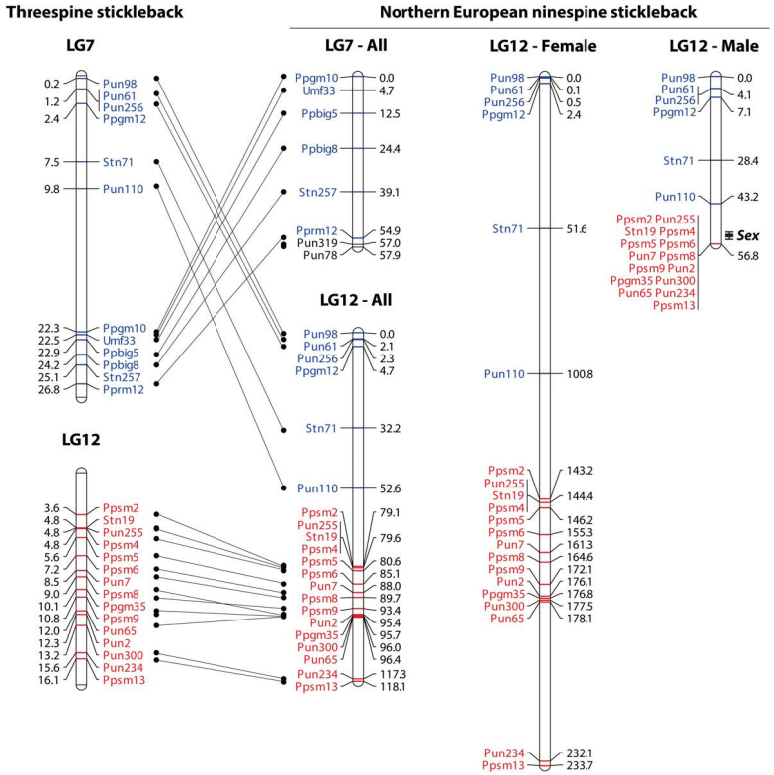


Figure 1 Synteny of LG7 and LG12 between the threespine and Northern European ninespine sticklebacks and sex-specific maps of LG12 in the Northern European ninespine stickleback. Marker positions are given in Mb for the threespine stickleback and in cM for the Northern European ninespine stickleback. Markers belonging to LG7 and LG12 in the threespine stickleback are indicated in blue and red, respectively. Location of the sex-determining locus (*Sex*) is shown in the male map with 95% confidence interval.

In the male meiosis, no recombination was observed between the 15 markers belonging to threespine stickleback LG12 (Figure 1) or between phenotypic sex and male-linked alleles at these loci (Table S4).

Mapping of pelvic reduction

In the F_2 progeny of F_1 hybrids (all F_1 fish had pelvic spines), pelvic spines were present in 218 individuals and absent in 65 individuals, in agreement with a 3:1 Mendelian ratio ($\chi^2 = 0.62$; $P = 0.43$). Mapping analyses detected a significant QTL for the lengths of left and right pelvic spines and girdles that exhibited extremely high *F*-values (196.1–309.6) and LOD scores (53.1–70.7) (Figure 2, Table 2). All of these traits were mapped to LG7 at 57 cM, the end of the LG (95% C.I. = 54–57 for left pelvic spine length and 56–57 for other traits) at marker Pun319, which is located within the *Pitx1* gene (Shapiro *et al.* 2009). This QTL region explained 58–69% of phenotypic variance in these traits (Table 2). In the analyses of different genotypes for three different alleles at Pun319, considerably lower mean values of these traits were observed for individuals with two alleles from Rytilampi than for those with other allelic combinations (ANOVA, $F_{3,267} = 118.3$–227.0; $P < 0.001$; Table 2). Individuals with alleles from both Helsinki and Rytilampi tended to show slightly lower mean values as compared to those with the homozygous alleles of Helsinki, exhibiting significant differences in four out of the eight comparisons (Tukey test, $P < 0.05$).

No significant or suggestive QTL were detected for these traits in other LGs, including LG4 (Figure 2), in which a strong QTL for these traits was identified in North American ninespine sticklebacks (Shapiro *et al.* 2009).

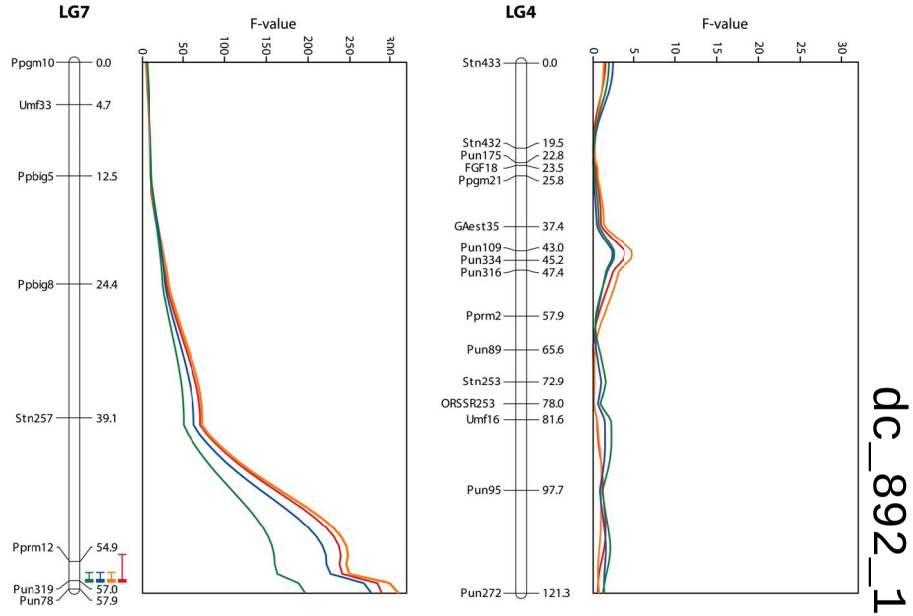


Figure 2 QTL location with 95% confidence interval and *F*-value for left (red and blue) and right (orange and green) pelvic spine (red and orange) and girdle (blue and green) lengths in LG7 and LG4. Marker positions are given in cM.

DISCUSSION

Genetic basis of parallel pelvic reduction

Our study uncovered a major genomic region determining pelvic reduction in Northern European ninespine sticklebacks. Based on the phenotypic segregation in the interpopulation cross, it is apparent that the reduction of pelvic spines is recessive and inherited in a simple Mendelian fashion, as observed in threespine sticklebacks (Cresko *et al.* 2004; Shapiro *et al.* 2004; Coyle *et al.* 2007). Both pelvic spine and girdle lengths were mapped to the same region where the *Pitx1* gene is located, explaining large proportions of the variance in these traits. This gene is known to be responsible for pelvic reduction in threespine sticklebacks (Cole *et al.* 2003; Cresko *et al.* 2004; Shapiro *et al.* 2004; Coyle *et al.* 2007; Chan *et al.* 2010). Thus, the *Pitx1* gene is a strong candidate for the determination of pelvic phenotypes in Northern European ninespine sticklebacks.

Shapiro *et al.* (2006) conducted interspecific crosses between threespine and ninespine sticklebacks to test whether the same gene or a different gene is responsible for pelvic reduction in these two species. Based on the phenotypes of parents and their F_1 hybrid progeny, they suggested that the same genetic mechanism is likely to be involved in pelvic reduction in the two species. However, in a mapping study of ninespine sticklebacks with the F_1 individuals of a cross between parents lacking pelvic structures from phenotypically monomorphic Canadian and polymorphic Alaskan populations, the major genomic region involved in pelvic reduction was identified on LG4, which was completely distinct from the *Pitx1* locus on LG7 (Shapiro *et al.* 2009). This region explained large proportions (81–87%) of the phenotypic variance of pelvic spine and girdle lengths in their cross, thus exhibiting a strong genetic impact on these traits (Shapiro *et al.* 2009). Given that the *Pitx1* gene appears to be involved in pelvic reduction in the Canadian population (Shapiro *et al.* 2006), it is likely that pelvic reduction is caused by different genes in these populations, implying that genetic variation resulting in similar phenotypes of pelvic structures exists in at least two different loci in North American ninespine sticklebacks. Based on our and previous studies (Shapiro *et al.* 2006, 2009), it appears that although the same genetic mechanism for pelvic reduction might have persisted in threespine and ninespine sticklebacks during 13 million years of divergence (Bell *et al.* 2009), an alternative genetic mechanism for pelvic reduction may have evolved in North American ninespine sticklebacks, possibly within the past 1.6 million years after the divergence from Northern European ninespine sticklebacks (Teacher *et al.* 2011) (Figure 3). Further mapping analyses with more populations from Northern Europe and North America should provide a more refined picture of the evolutionary process of different genetic architectures for pelvic reduction in sticklebacks.

It has been suggested that the likelihood of genetic parallelism underlying similar phenotypic changes decreases with increasing evolutionary distance between taxa (Elmer and Meyer 2011; Conte *et al.* 2012). There are several known cases in which the same genes

Table 2 QTL and phenotypic variation of pelvic spine and girdle lengths

Trait	LG	Position in cM (C.I.)	F	LOD	PVE (%)	Closest Marker	Phenotypic Mean (± SE) for Each Genotype at Pun319 (mm) HEL/HEL (N = 70)	HEL/RYT1 (N = 60)	HEL/RYT2 (N = 73)	RYT1/RYT2 (N = 68)
Left pelvic spine	LG7	57 (54–57)	288.34	67.80	67	Pun319	3.62 ± 0.09	3.22 ± 0.11	3.41 ± 0.07	0.47 ± 0.13
Right pelvic spine	LG7	57 (56–57)	309.61	70.73	69	Pun319	3.49 ± 0.09	3.23 ± 0.11	3.32 ± 0.07	0.35 ± 0.12
Left pelvic girdle	LG7	57 (56–57)	275.72	65.99	66	Pun319	7.98 ± 0.13	7.44 ± 0.16	7.41 ± 0.09	3.90 ± 0.18
Right pelvic girdle	LG7	57 (56–57)	196.05	53.11	58	Pun319	7.61 ± 0.13	7.14 ± 0.16	7.11 ± 0.08	4.37 ± 0.17

C.I., 95% confidence interval; PVE, proportion of phenotypic variation explained; HEL, allele from Helsinki; RYT, allele from Rytilampi.

are responsible for phenotypic parallelism within the same or closely related species (Wood *et al.* 2005; Arendt and Reznick 2008; Nadeau and Jiggins 2010). However, the same gene can underlie similar phenotypes even across disparate taxonomic groups (Arendt and Reznick 2008; Nadeau and Jiggins 2010; Conte *et al.* 2012). The *Melanocortin 1 receptor* (*Mc1r*) gene is a case in point; it is known to control for pigmentation variation in diverse vertebrate taxa, including mammals, birds, and reptiles (Manceau *et al.* 2010). The most probable form of genetic parallelism among distantly related taxa is provided by repeated independent mutations in the same gene rather than shared ancestral genetic variation (Elmer and Meyer 2011). Thus far, empirical studies to infer parallel genetic evolution have been mostly based on candidate gene approaches, which rely on *a priori* hypothesis with respect to coding sequence or expression differences in a particular gene of interest (Wood *et al.* 2005; Arendt and Reznick 2008; Nadeau and Jiggins 2010). Consequently, inferences about different genetic mechanisms can be made solely on the basis of the lack of evidence for genetic parallelism in candidate genes. In contrast, genetic mapping provides powerful means to identify the locations and magnitudes of genomic regions controlling various phenotypic traits on a genome-wide scale. Despite the fact that intraspecific comparative mapping has been rarely performed—partly because of logistical limitations to establish mapping crosses—a few recent studies have begun to identify different genes or genomic regions involved in similar phenotypes within the same species (Gross *et al.* 2009; Thurber *et al.* 2013) as well as between closely related species (Ng *et al.* 2008; Lee *et al.* 2011). Although generalizations about the genetic mechanisms and evolutionary processes that lead to similar phenotypes may as yet be too early (Kopp 2009; Elmer and Meyer 2011), the case of pelvic reduction in ninespine sticklebacks highlights the fact that alternative QTL resulting in similar phenotypes can evolve over a short evolutionary time scale. This emphasizes the importance of both interspecific and intraspecific comparisons in understanding the genetic architectures of parallel phenotypic evolution.

In addition to a major genomic region explaining 81–87% of the variance in pelvic structures, a modifier locus of smaller effect was mapped to LG1 in North American ninespine sticklebacks (Shapiro *et al.* 2009). Similarly, four modifier loci were identified in threespine sticklebacks in which the *Pitx1* locus explained 65% and 47% of the variance in pelvic spine and girdle lengths, respectively (Shapiro *et al.* 2004). Although the *Pitx1* locus explained similar proportions of the variance in these traits in Northern European ninespine sticklebacks as observed in threespine sticklebacks (Shapiro *et al.* 2004), no significant modifier loci were detected in our study. Based on these results, it is likely that the number and location of modifier loci with small effects can differ between populations or species in sticklebacks, possibly because of their different genetic backgrounds. It should be also noted that the possible existence of modifier loci with small effects in the European lineage cannot be excluded because the power of QTL mapping largely depends on the numbers of individuals and loci screened (Hu and Xu 2008; Slate *et al.* 2010). Nevertheless, it is worth noticing that the numbers of progeny (283 *vs.* 120) and loci (226 *vs.* 190) screened in this study were larger than those in the study by Shapiro *et al.* (2009).

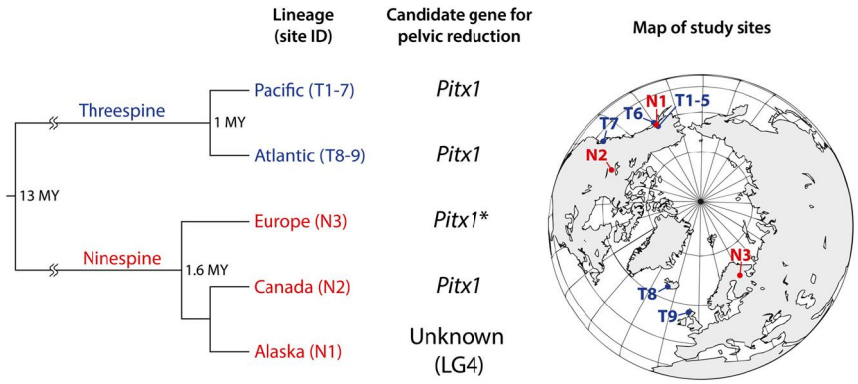


Figure 3 Phylogenetic relationships and candidate genes for pelvic reduction in threespine and ninespine sticklebacks. The phylogeny is based on molecular (Haglund *et al.* 1992b; Ortí *et al.* 1994; Aldenhoven *et al.* 2010; Teacher *et al.* 2011) and fossil data (Bell *et al.* 2009). The candidate genes were determined by the previous (Cole *et al.* 2003; Cresko *et al.* 2004; Shapiro *et al.* 2004, 2006, 2009; Coyle *et al.* 2007; Chan *et al.* 2010) and current* studies.

Closely related populations are expected to have the same or similar genetic bases of parallel phenotypic changes because of their similar genetic backgrounds and selection trajectories (Wood *et al.* 2005; Barrett and Schluter 2008; Pritchard *et al.* 2010). In threespine sticklebacks, rapid morphological transitions after freshwater colonization have occurred in fish belonging to the Pacific and Atlantic clades, which diverged approximately one million years ago (Haglund *et al.* 1992b; Ortí *et al.* 1994; Higuchi and Goto 1996). Based on genetic analyses with globally distributed populations, it appears that the reductions of lateral plates and pelvic structures have evolved via the same genetic mechanisms in the different lineages, implying that the ancestral marine threespine sticklebacks of these lineages shared the same or similar genetic variation for these traits (Cresko *et al.* 2004; Shapiro *et al.* 2004; Colosimo *et al.* 2005; Coyle *et al.* 2007). However, even though the same major gene is responsible for the reduction of lateral plates in a number of freshwater populations, an exception was found in one Japanese population, which is located in the southernmost area of their distribution range (Colosimo *et al.* 2005). This population is thought to have colonized freshwater approximately 40,000 years ago during the middle Pleistocene period (Watanabe *et al.* 2003), whereas freshwater colonization in other populations has occurred mostly within the past 12,000 years in postglacial times (Bell and Foster 1994). Hence, the different colonization histories might be associated with the occurrence of an alternative genetic mechanism for lateral plate reduction. In contrast to threespine sticklebacks, ninespine sticklebacks comprise several divergent lineages, providing more potential for their independent evolution (Haglund *et al.* 1992a; Takahashi and Goto 2001; Aldenhoven *et al.* 2010; Shikano *et al.* 2010a). Therefore, the opportunity to evolve alternative genetic mechanisms for similar phenotypes may be increased, as found in the case of pelvic reduction of this species. Further interspecific and intraspecific comparative studies with sticklebacks may provide important insights into the genetic underpinnings of parallel phenotypic evolution and the factors underlying the occurrence of different genetic mechanisms that lead to similar phenotypes.

Chromosomal rearrangements

The comparative mapping revealed that a segment of one autosome corresponding to LG7 in threespine sticklebacks is linked to LG12 in Northern European ninespine sticklebacks. Our results also indicated that none of the loci located on this segmental part shows linkage to the markers mapped to LG7. Hence, the rearrangement of genetic linkage patterns is likely attributable to a chromosomal rearrangement that has occurred after the divergence between threespine and ninespine sticklebacks. Because no linkage was detected between the segmental part and either the remaining region of LG7 or LG12 in the genetic map of North American ninespine sticklebacks (Shapiro *et al.* 2009), it is not certain if the arrangement of linkage patterns has occurred before or after the split between Northern European and North American populations. Our study also identified possible chromosomal inversions in several LGs as compared to the threespine stickleback genome, although potential errors in the genome sequences cannot be ruled out (Ross and Peichel 2008; Natri *et al.* 2013). Further cytogenetic analyses would clarify whether the linkage between LG12 and the segment of LG7 has formed via physical or pseudo linkage, as well as verify the occurrence of intrachromosomal rearrangements in Northern European ninespine sticklebacks.

Although rapid turnover of sex chromosome systems is often observed in fish, including sticklebacks (Devlin and Nagahama 2002; Mank and Avise 2009), the sex-determining gene was mapped to LG12 in Northern European ninespine sticklebacks, as in North American fish (Shapiro *et al.* 2009). In the male meiosis of both North American and Northern European ninespine sticklebacks, no recombination was observed in the chromosomal region corresponding to threespine stickleback LG12. Nevertheless, it is noteworthy that the interchromosomal rearrangement of linkage patterns was found for the sex chromosomes in our study. Cytogenetic analyses have shown that the Y chromosome of Northern European and North American ninespine sticklebacks is much larger than the X chromosome because of a Y chromosome rearrangement, possibly as a result of a duplication of the ancestral Y chromosome or an insertion of a duplicated autosomal segment into the Y chromosome (Ocalewicz *et al.* 2008; Ross *et al.* 2009). However, because the linkage between LG12 and the segment of LG7 was identified both in female and male maps in our study, it is unlikely that the Y chromosome rearrangement is a proximate cause of the rearrangement of linkage patterns. Although it is not certain whether the linkage between LG12 and the segment of LG7 is caused by physical or pseudo linkage, it appears that the segmental region of LG7 co-segregates with the sex-determining locus in Northern European ninespine sticklebacks. Theoretical studies have shown that the formation of linkage between the sex-determining locus and autosomal genes under sexually antagonistic selection has significant consequences on both population fitness and sex chromosome evolution (Charlesworth and Charlesworth 1980; van Doorn and Kirkpatrick 2007). As such, it would be of particular interest to assess if genes underlying sexually dimorphic traits and mating behavior reside in the chromosomal region newly linked to the sex-determining locus.

CONCLUSIONS

Our study demonstrated that the *Pitx1* gene is a strong candidate for the determination of pelvic reduction in Northern European ninespine sticklebacks. The interspecific and intraspecific comparative analyses indicated that although the same genetic mechanism for pelvic reduction might have persisted in threespine and ninespine sticklebacks, alternative QTL for pelvic reduction might have evolved in ninespine sticklebacks, possibly within the past 1.6 million years after the split between Northern European and North American populations. Hence, our study gives empirical support for the view that alternative genetic mechanisms leading to similar phenotypes can evolve over a relatively short evolutionary time scale.

ACKNOWLEDGMENTS

We thank Abigél Gonda and Mirva Turtiainen for help with the fish breeding, and Kirsi Kähkönen and Marika Karjalainen for assistance in the laboratory. We also thank Scott McCairns for comments on an earlier version of the manuscript. Our research was supported by the Academy of Finland (grants 129662, 134728, and 218343 to J.M. and grant 128716 to G.H.) and by the Biological Interactions Graduate School (V.N.L.).

LITERATURE CITED

Aldenhoven, J. T., M. A. Miller, P. S. Corneli, and M. D. Shapiro, 2010 Phylogeography of ninespine sticklebacks (*Pungitius pungitius*) in North America: glacial refugia and the origins of adaptive traits. Mol. Ecol. 19: 4061–4076.

Arendt, J., and D. Reznick, 2008 Convergence and parallelism reconsidered: what have we learned about the genetics of adaptation? Trends Ecol. Evol. 23: 26–32.

Barrett, R. D., and D. Schluter, 2008 Adaptation from standing genetic variation. Trends Ecol. Evol. 23: 38–44.

Bell, M. A., 1976 Evolution of phenotypic diversity in *Gasterosteus aculeatus* superspecies on the Pacific coast of North America. Syst. Zool. 25: 211–227.

Bell, M. A., and S. A. Foster, 1994 Introduction to the evolutionary biology of the threespine stickleback, pp. 1–27 in *The Evolutionary Biology of the Threespine Stickleback*, edited by M. A. Bell, and S. A. Foster. Oxford University Press, Oxford.

Bell, M. A., J. D. Stewart, and P. J. Park, 2009 The world's oldest fossil threespine stickleback fish. Copeia 2009: 256–265.

Bookstein, F. L., 1991 *Morphometric Tools for Landmark Data: Geometry and Biology*, Cambridge University Press, Cambridge.

Chan, Y. F., M. E. Marks, F. C. Jones, G. Villarreal, Jr., M. D. Shapiro *et al.*, 2010 Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a *Pitx1* enhancer. Science 327: 302–305.

Charlesworth, D., and B. Charlesworth, 1980 Sex differences in fitness and selection for centric fusions between sex-chromosomes and autosomes. Genet. Res. 35: 205–214.

Cioffi, M. B., O. Moreira Filho, L. F. Almeida-Toledo, and L. A. C. Bertollo, 2012 The contrasting role of heterochromatin in the differentiation of sex chromosomes: an overview from Neotropical fish. J. Fish Biol. 80: 2125–2139.

Cole, N. J., M. Tanaka, A. Prescott, and C. A. Tickle, 2003 Expression of limb initiation genes and clues to the basis of morphological diversification in threespine sticklebacks. Curr. Biol. 13: R951–R952.

Colosimo, P. F., C. L. Peichel, K. Nereng, B. K. Blackman, S. D. Shapiro *et al.*, 2004 The genetic architecture of parallel armor plate reduction in threespine sticklebacks. PLoS Biol. 2: 635–641.

Colosimo, P. F., K. E. Hosemann, S. Balabhadra, G. Villarreal, Jr., M. Dickson *et al.*, 2005 Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. Science 307: 1928–1933.

Conte, G. L., M. E. Arnegard, C. L. Peichel, and D. Schluter, 2012 The probability of genetic parallelism and convergence in natural populations. Proc. Biol. Sci. 279: 5039–5047.

Coyle, S. M., F. A. Huntingford, and C. L. Peichel, 2007 Parallel evolution of *Pitx1* underlies pelvic reduction in Scottish threespine stickleback (*Gasterosteus aculeatus*). J. Hered. 98: 581–586.

Cresko, W. A., A. Amores, C. Wilson, J. Murphy, M. Currey *et al.*, 2004 Parallel genetic basis for repeated evolution of armor loss in Alaskan threespine stickleback populations. Proc. Natl. Acad. Sci. USA 101: 6050–6055.

Devlin, R. H., and Y. Nagahama, 2002 Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208: 191–364.

Elmer, K. R., and A. Meyer, 2011 Adaptation in the age of ecological genomics: insights from parallelism and convergence. Trends Ecol. Evol. 26: 298–306.

Elphinstone, M. S., G. N. Hinten, M. J. Anderson, and C. J. Nock, 2003 An inexpensive and high-throughput procedure to extract and purify total genomic DNA for population studies. Mol. Ecol. Notes 3: 317–320.

Endler, J. A., 1986 *Natural Selection in the Wild*, Princeton University Press, Princeton.

Ferguson-Smith, M. A., and V. Trifonov, 2007 Mammalian karyotype evolution. Nat. Rev. Genet. 8: 950–962.

Green, P., K. Falls, and S. Crooks, 1990 *Documentation for CRI-MAP (version 2.4)*, Washington University School of Medicine, St. Louis.

Gross, J. B., R. Borowsky, and C. J. Tabin, 2009 A novel role for *Mc1r* in the parallel evolution of depigmentation in independent populations of the cavefish *Astyanax mexicanus*. PLoS Genet. 5: e1000326.

Haglund, T. R., D. G. Buth, and R. Lawson, 1992a Allozyme variation and phylogenetic relationships of Asian, North American, and European populations of the ninespine stickleback, *Pungitius pungitius*, pp. 438–452 in *Systematics, Historical Ecology, and North American Freshwater Fishes*, edited by R. L. Mayden Stanford University Press, Stanford.

Haglund, T. R., D. G. Buth, and R. Lawson, 1992b Allozyme variation and phylogenetic relationships of Asian, North American, and European populations of the threespine stickleback. Copeia 1992: 432–443.

Heckel, G., M. Zbinden, D. Mazzi, A. Kohler, G. Reckeweg *et al.*, 2002 Microsatellite markers for the three-spined stickleback (*Gasterosteus aculeatus* L.) and their applicability in a freshwater and an anadromous population. Conserv. Genet. 3: 79–81.

Herczeg, G., M. Turtiainen, and J. Merilä, 2010 Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. Biol. J. Linn. Soc. Lond. 101: 403–416.

Higuchi, M., and A. Goto, 1996 Genetic evidence supporting the existence of two distinct species in the genus *Gasterosteus* around Japan. Environ. Biol. Fishes 47: 1–16.

Hu, Z., and S. Xu, 2008 A simple method for calculating the statistical power for detecting a QTL located in a marker interval. Heredity 101: 48–52.

Huey, R. B., G. W. Gilchrist, M. L. Carlson, D. Berrigan, and L. Serra, 2000 Rapid evolution of a geographic cline in size in an introduced fly. Science 287: 308–309.

Hufton, A. L., D. Groth, M. Vingron, H. Lehrach, A. J. Poustka *et al.*, 2008 Early vertebrate whole genome duplications were predated by a period of intense genome rearrangement. Genome Res. 18: 1582–1591.

Huttley, G. A., M. J. Wakefield, and S. Easteal, 2007 Rates of genome evolution and branching order from whole genome analysis. Mol. Biol. Evol. 24: 1722–1730.

Jaillon, O., J. M. Aury, F. Brunet, J. L. Petit, N. Stange-Thomann *et al.*, 2004 Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. Nature 431: 946–957.

Jeffery, W. R., 2009 Regressive evolution in *Astyanax* cavefish. Annu. Rev. Genet. 43: 25–47.

Jones, R., D. C. Culver, and T. C. Kane, 1992 Are parallel morphologies of cave organisms the result of similar selection pressures? Evolution 46: 353–365.

Jones, F. C., M. G. Grabherr, Y. F. Chan, P. Russell, E. Mauceli *et al.*, 2012 The genomic basis of adaptive evolution in threespine sticklebacks. Nature 484: 55–61.

Kingsley, D. M., and C. L. Peichel, 2007 The molecular genetics of evolutionary change in sticklebacks, pp. 41–81 in *Biology of the Three-Spined Stickleback*, edited by Östlund-Nilsson S., I. Mayer, and F. A. Huntingford. CRC Press, Boca Raton.

Kitano, J., and C. L. Peichel, 2012 Turnover of sex chromosomes and speciation in fishes. Environ. Biol. Fishes 94: 549–558.

Kohn, M., J. Högel, W. Vogel, P. Minich, H. Kehrer-Sawatzki *et al.*, 2006 Reconstruction of a 450-My-old ancestral vertebrate protokaryotype. Trends Genet. 22: 203–210.

Kopp, A., 2009 Metamodels and phylogenetic replication: a systematic approach to the evolution of developmental pathways. Evolution 63: 2771–2789.

Laine, V. N., C. R. Primmer, G. Herczeg, J. Merilä, and T. Shikano, 2012 Isolation and characterization of 13 new nine-spined stickleback, *Pungitius pungitius*, microsatellites located nearby candidate genes for behavioural variation. Ann. Zool. Fenn. 49: 123–128.

Landry, L., W. F. Vincent, and L. Bernatchez, 2007 Parallel evolution of lake whitefish dwarf ecotypes in association with limnological features of their adaptive landscape. J. Evol. Biol. 20: 971–984.

Largiadèr, C. R., V. Fries, B. Kobler, and T. C. M. Bakker, 1999 Isolation and characterization of microsatellite loci from the three-spined stickleback (*Gasterosteus aculeatus* L.). Mol. Ecol. 8: 342–344.

Lee, S. F., L. Rako, and A. A. Hoffmann, 2011 Genetic mapping of adaptive wing size variation in *Drosophila simulans*. Heredity 107: 22–29.

Losos, J. B., T. R. Jackman, A. Larson, K. de Queiroz, and L. Rodríguez-Schettino, 1998 Historical contingency and determinism in replicated adaptive radiations of island lizards. Science 279: 2115–2118.

Mäkinen, H. S., J. M. Cano, and J. Merilä, 2008 Identifying footprints of directional and balancing selection in marine and freshwater three-spined stickleback (*Gasterosteus aculeatus*) populations. Mol. Ecol. 17: 3565–3582.

Manceau, M., V. S. Domingues, C. R. Linnen, E. B. Rosenblum, and H. E. Hoekstra, 2010 Convergence in pigmentation at multiple levels: mutations, genes and function. Phil. Trans. R. Soc. B. 365: 2439–2450.

Mank, J. E., and J. C. Avise, 2009 Evolutionary diversity and turn-over of sex determination in teleost fishes. Sex Dev. 3: 60–67.

Miller, C. T., S. Beleza, A. A. Pollen, D. Schluter, R. A. Kittles *et al.*, 2007 *cis*-Regulatory changes in *Kit ligand* expression and parallel evolution of pigmentation in sticklebacks and humans. Cell 131: 1179–1189.

Merilä, J., 2013 Nine-spined stickleback (*Pungitius pungitius*): an emerging model for evolutionary biology research. Ann. N. Y. Acad. Sci. 1289: 18–35.

Nadeau, N. J., and C. D. Jiggins, 2010 A golden age for evolutionary genetics? Genomic studies of adaptation in natural populations. Trends Genet. 26: 484–492.

Naruse, K., M. Tanaka, K. Mita, A. Shima, J. Postlethwait *et al.*, 2004 A medaka gene map: the trace of ancestral vertebrate proto-chromosomes revealed by comparative gene mapping. Genome Res. 14: 820–828.

Natri, H. M., T. Shikano, and J. Merilä, 2013 Progressive recombination suppression and differentiation in recently evolved neo-sex chromosomes. Mol. Biol. Evol. 30: 1131–1144.

Ng, C. S., A. M. Hamilton, A. Frank, O. Barmina, and A. Kopp, 2008 Genetic basis of sex-specific color pattern variation in *Drosophila malerkotliana*. Genetics 180: 421–429.

Ocalewicz, K., D. Fopp-Bayat, P. Woznicki, and M. Jankun, 2008 Heteromorphic sex chromosomes in the ninespine stickleback *Pungitius pungitius*. J. Fish Biol. 73: 456–462.

Ortí, G., M. A. Bell, T. E. Reimchen, and A. Meyer, 1994 Global survey of mitochondrial-DNA sequences in the threespine stickleback: evidence for recent migrations. Evolution 48: 608–622.

Östlund-Nilsson, S., and I. Mayer, 2007 The biology of other sticklebacks, pp. 353–372 in *Biology of the Three-Spined Stickleback*, edited by Östlund-Nilsson S., I. Mayer, and F. A. Huntingford. CRC Press, Boca Raton.

Peichel, C. L., K. Nereng, K. A. Ohgi, B. L. E. Cole, P. F. Colosimo *et al.*, 2001 The genetic architecture of divergence between threespine stickleback species. Nature 414: 901–905.

Pritchard, J. R., and D. Schluter, 2001 Declining interspecific competition during character displacement: summoning the ghost of competition past. Evol. Ecol. Res. 3: 209–220.

Pritchard, J. K., J. K. Pickrell, and G. Coop, 2010 The genetics of human adaptation: hard sweeps, soft sweeps, and polygenic adaptation. Curr. Biol. 20: R208–R215.

Ravi, V., and B. Venkatesh, 2008 Rapidly evolving fish genomes and teleost diversity. Curr. Opin. Genet. Dev. 18: 544–550.

Reznick, D. N., F. H. Rodd, and M. Cardenas, 1996 Life-history evolution in guppies (*Poecilia reticulata*: Poeciliidae). 4. Parallelism in life-history phenotypes. Am. Nat. 147: 319–338.

Rogers, S. M., and L. Bernatchez, 2007 The genetic architecture of ecological speciation and the association with signatures of selection in natural lake whitefish (*Coregonus* sp. Salmonidae) species pairs. Mol. Biol. Evol. 24: 1423–1438.

Rohlf, F. J., 2006 *TpsDig version 2.10*, Department of Ecology and Evolution, State University of New York, Stony Brook, New York.

Ross, J. A., and C. L. Peichel, 2008 Molecular cytogenetic evidence of rearrangements on the Y chromosome of threespine stickleback fish. Genetics 179: 2173–2182.

Ross, J. A., J. R. Urton, J. Boland, M. D. Shapiro, and C. L. Peichel, 2009 Turnover of sex chromosomes in the stickleback fishes (Gasterosteidae). PLoS Genet. 5: e1000391.

Schluter, D., 2000 *The ecology of adaptive radiation*, Oxford University Press, Oxford.

Seaton, G., J. Hernandez, J. Grunchec, I. White, J. Allen *et al.*, 2006 GridQTL: a grid portal for QTL mapping of compute intensive datasets. Proceedings of the 8th World Congress on Genetics Applied to Livestock. Belo Horizonte, Brazil.

Sémon, M., and K. H. Wolfe, 2007 Rearrangement rate following the whole-genome duplication in teleosts. Mol. Biol. Evol. 24: 860–867.

Shapiro, M. D., M. E. Marks, C. L. Peichel, B. K. Blackman, K. S. Nereng *et al.*, 2004 Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature 428: 717–723.

Shapiro, M. D., M. A. Bell, and D. M. Kingsley, 2006 Parallel genetic origins of pelvic reduction in vertebrates. Proc. Natl. Acad. Sci. USA 103: 13753–13758.

Shapiro, M. D., B. R. Summers, S. Balabhadra, A. L. Miller, C. B. Cunningham *et al.*, 2009 The genetic architecture of skeletal convergence and sex determination in ninespine sticklebacks. Curr. Biol. 19: 1140–1145.

Shikano, T., Y. Shimada, G. Herczeg, and J. Merilä, 2010a History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. Mol. Ecol. 19: 1147–1161.

Shikano, T., J. Ramadevi, Y. Shimada, and J. Merilä, 2010b Utility of sequenced genomes for microsatellite marker development in non-model organisms: a case study of functionally important genes in nine-spined sticklebacks (*Pungitius pungitius*). BMC Genomics 11: 334.

Shikano, T., H. M. Natri, Y. Shimada, and J. Merilä, 2011 High degree of sex chromosome differentiation in stickleback fishes. BMC Genomics 12: 474.

Shimada, Y., T. Shikano, and J. Merilä, 2011 A high incidence of selection on physiologically important genes in the three-spined stickleback, *Gasterosteus aculeatus*. Mol. Biol. Evol. 28: 181–193.

Slate, J., A. W. Santure, P. G. D. Feulner, E. A. Brown, A. D. Ball *et al.*, 2010 Genome mapping in intensively studied wild vertebrate populations. Trends Genet. 26: 275–284.

Takahashi, H., and A. Goto, 2001 Evolution of East Asian ninespine sticklebacks as shown by mitochondrial DNA control region sequences. Mol. Phylogenet. Evol. 21: 135–155.

Teacher, A. G. F., T. Shikano, M. E. Karjalainen, and J. Merilä, 2011 Phylogeography and genetic structuring of European nine-spined sticklebacks (*Pungitius pungitius*) - mitochondrial DNA evidence. PLoS ONE 6: e19476.

Thurber, C. S., M. H. Jia, Y. Jia, and A. L. Caicedo, 2013 Similar traits, different genes? Examining convergent evolution in related weedy rice populations. Mol. Ecol. 22: 685–698.

van Doorn, G., and M. Kirkpatrick, 2007 Turnover of sex chromosomes induced by sexual conflict. Nature 449: 909–912.

Voorrips, R. E., 2002 MapChart: Software for the graphical presentation of linkage maps and QTLs. J. Hered. 93: 77–78.

Walker, J. A., and M. A. Bell, 2000 Net evolutionary trajectories of body shape evolution within a microgeographic radiation of threespine sticklebacks (*Gasterosteus aculeatus*). J. Zool. (Lond.) 252: 293–302.

Watanabe, K., S. Mori, and M. Nishida, 2003 Genetic relationships and origin of two geographic groups of the freshwater threespine stickleback, 'Hariyo'. Zoolog. Sci. 20: 265–274.

Wood, T. E., J. K. Burke, and L. H. Rieseberg, 2005 Parallel genotypic adaptation: when evolution repeats itself. Genetica 123: 157–170.

Woods, I. G., C. Wilson, B. Friedlander, P. Chang, D. K. Reyes *et al.*, 2005 The zebrafish gene map defines ancestral vertebrate chromosomes. Genome Res. 15: 1307–1314.

Wootton, R. J., 1976 *The Biology of the Sticklebacks*, Academic Press, London.

Zhou, H., N. Deeb, C. M. Evock-Clover, C. M. Ashwell, and S. J. Lamont, 2006 Genome-wide linkage analysis to identify chromosomal regions affecting phenotypic traits in the chicken. I. Growth and average daily gain. Poult. Sci. 85: 1700–1711.

Communicating editor: B. J. Andrews

JOURNAL OF Evolutionary Biology

doi: 10.1111/j.1420-9101.2011.02286.x

Intraspecific divergence in the lateral line system in the nine-spined stickleback (*Pungitius pungitius*)

N. TROKOVIC, G. HERCZEG, R. J. SCOTT McCAIRNS, N. IZZA AB GHANI & J. MERILÄ

Department of Biosciences, Ecological Genetics Research Unit, University of Helsinki, Helsinki, Finland

Keywords:

geographic variation;
lateral line;
neuromast;
P_{ST};
Pungitius pungitius;
sensory system.

Abstract

The mechanosensory lateral line system of fishes is an important organ system conveying information crucial to individual fitness. Yet, our knowledge of lateral line diversity is almost exclusively based on interspecific studies, whereas intraspecific variability and possible population divergence have remained largely unexplored. We investigated lateral line system variability in four marine and five pond populations of nine-spined stickleback (*Pungitius pungitius*). We found significant differences in neuromast number between pond and marine fish. In particular, three of seventeen lateral line regions (*viz.* caudal peduncle superficial neuromasts; canal neuromasts from the anterior trunk and caudal peduncle) showed strong divergence between habitats. Similar results were obtained with laboratory-reared individuals from a subset of populations, suggesting that the patterns found in nature likely have a genetic basis. Interestingly, we also found habitat-dependent population divergence in neuromast variability, with pond populations showing greater heterogeneity than marine populations, although only in wild-caught fish. A comparison of neutral genetic (F_{ST}) and phenotypic (P_{ST}) differentiation suggested that natural selection is likely associated with habitat-dependent divergence in neuromast counts. Hence, the results align with the conclusion that the mechanosensory lateral line system divergence among marine and pond nine-spined sticklebacks is adaptive.

Introduction

Spatially varying selection pressures are expected to drive both phenotypic and genotypic divergence among populations of the same species (Mayr, 1963; Endler, 1977; Conover & Schultz, 1995). Populations residing in different habitats may be faced with markedly different biotic and abiotic environmental conditions, which may put different demands on sensory systems and, thus, lead to population differentiation in these systems (e.g. McClelland *et al.*, 1998; Guillén *et al.*, 2000; Endler *et al.*, 2001; Fuller *et al.*, 2003; Dangles *et al.*, 2005). The mechanosensory lateral line system of fishes and aquatic amphibians responds to weak water movements and is involved in prey detection (Hoekstra & Janssen, 1985; Saunders & Montgomery, 1985; Montgomery & Macdonald, 1987; Montgomery & Milton, 1993), schooling behaviour (Partridge & Pitcher, 1980), rheotaxis (Montgomery *et al.*, 1997), as well as avoidance of predators (Blaxter & Fuiman, 1990; Fuiman, 1993) and obstacles (Campenhausen *et al.*, 1981; Teyke, 1985). Functional units of the lateral line system, called neuromasts, are distributed either superficially over the body surface (superficial neuromasts) or in fluid-filled canals (canal neuromasts). Experimental studies have shown that superficial neuromasts are sensitive to water velocity (Van Netten & Kroese, 1987; Kalmijn, 1988) and mediate behaviours such as rheotaxis (Montgomery *et al.*, 1997), whereas canal neuromasts are sensitive to water acceleration (Kalmijn, 1988; Engelmann *et al.*, 2000) and mediate schooling (Cahn *et al.*, 1968; Gallego & Heath, 1994), short-distance prey detection and localization of objects (Coombs *et al.*, 2000; Coombs *et al.*, 2001).

Lateral line system abundance and distribution vary widely among species (Coombs *et al.*, 1988; Webb, 1989).

Correspondence: Nina Trokovic, Ecological Genetics Research Unit, Department of Biosciences, PO Box 65, FI-00014 University of Helsinki, Finland.
Tel.: +358 9 191 57802; fax: +358 9 191 57694;
e-mail: nina.trokovic@helsinki.fi

Associations between structure of the lateral line system and ecological factors across species suggest that variation in the lateral line system may reflect adaptation to different ecological conditions. For example, blind cavefish and fish species with nocturnal feeding have increased number of neuromasts (Montgomery *et al.*, 2001; Carton & Montgomery, 2004). In fish species that are slow or infrequent swimmers, or those inhabiting lentic environments, superficial neuromasts are more abundant than canal neuromasts, whereas canal neuromasts predominate in species that are fast swimmers, highly active, or inhabit environments with constant water flow (Webb, 1989; Montgomery *et al.*, 1995; Engelmann *et al.*, 2000).

Our knowledge of lateral line system diversity is almost exclusively based on interspecific studies (e.g. Coombs *et al.*, 1988; Webb, 1989; Maruska, 2001). Yet despite the heuristic potential in studies of spatially divergent populations, scant interest has been paid to intraspecific comparisons, though see Michel *et al.* (2008) for a restricted analysis of the oculoscapular canals of common bully (*Gobiomorphus cotidianus*). More recently, researchers have demonstrated variation in superficial neuromast numbers among ecologically divergent three-spined stickleback populations (*Gasterosteus aculeatus*; Wark & Peichel, 2010).

The nine-spined stickleback (*Pungitius pungitius*) is a small teleost fish with a circumpolar distribution which inhabits a wide array of environments ranging from marine to freshwater, and fluvial to lacustrine habitats (Bănărescu & Paepke, 2001). In comparison with three-spined sticklebacks, the lateral line system of nine-spined sticklebacks appears to be more complex, containing both superficial and canal neuromasts (Honkanen, 1993). Moreover, the species displays marked morphological and behavioural variation in relation to the diverse habitat types in which it can be found. For example, pond fish are larger (Herczeg *et al.*, 2009a, 2010a), quicker in feeding, more aggressive and bolder (Herczeg *et al.*, 2009b), face higher costs of group living (Gonda *et al.*, 2009a; Herczeg *et al.*, 2009c), have reduced/lost body armour together with divergent body shape (Herczeg *et al.*, 2010b) and have different brain architecture (Gonda *et al.*, 2009b) as compared to their marine conspecifics. Hence, considering their complex lateral line system, in addition to the extensive divergence in morphological, behavioural and life history traits, the nine-spined stickleback is an excellent model system for studying population divergence of the lateral line system.

The aim of this study was to investigate population differentiation in the number of canal and superficial neuromasts with respect to habitat type among four marine and five pond populations of nine-spined sticklebacks. Large differences in both biotic (predators, competitors) and abiotic (structural heterogeneity, currents) environmental complexity between the two habitat types could result in habitat-specific population divergence in the lateral line system. Associations between neuromast abundance and habitat characteristics have been detected in interspecific studies (Webb, 1989; Montgomery *et al.*, 1995; Engelmann *et al.*, 2000), and on this basis, we predicted that fish in marine populations – living in a more complex habitat – would have larger numbers of neuromasts than fish in pond populations. We also investigated possible sexual dimorphism in the lateral line system, as behavioural and life history specializations between sexes (e.g. Herczeg *et al.*, 2010a) may have selected for sex differences in the structure and organisation of the lateral line system. Using F1 progeny from a smaller set of populations reared under controlled laboratory settings, we investigated whether patterns found in nature had a genetic basis. Finally, by comparing neutral genetic variation – as reflected in F_{ST} estimated on the basis of variability in 23 microsatellite loci – with an index of phenotypic divergence (P_{ST}; Leinonen *et al.*, 2006; Saether *et al.*, 2007) in wild populations, we tested whether habitat-based divergence in the lateral line system could be explained by drift alone, or whether natural selection could be invoked to explain the observed degree of differentiation.

Materials and methods

Study species and populations

We collected adult nine-spined sticklebacks during the breeding season (May and June) of 2007–2009 from five ponds and four marine populations from geographically distinct locations in Fennoscandia (Fig. 1; Table 1). The

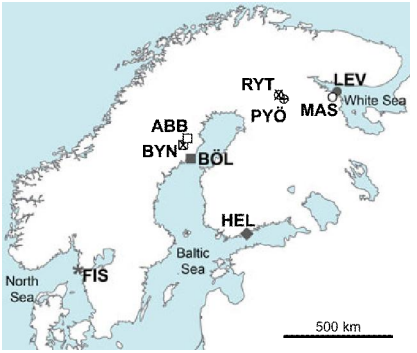


Fig. 1 Map depicting the nine sampling localities. Marine sites are indicated as solid, grey symbols while open, black symbols denote freshwater populations. Sites are denoted with a unique symbol, also used in subsequent ordination plots to facilitate identification of sample origins.

Table 1 Sampled nine-spined stickleback populations with information about their geographical location, habitat type and sample sizes. M = male, F = female.

Population	Abbreviation	Habitat type	Coordinates	Sample sizes		
				M	F	All
Bölesviken, Baltic Sea (Sweden)	BÖL	Marine	63°39′N; 20°12′E	10	10	20
Helsinki, Baltic Sea (Finland)	HEL	Marine	60°13′N; 25°11′E	20	0	20
Levin Navolok, White Sea (Russia)	LEV	Marine	66°18′N; 33°25′E	20	0	20
Fiskebäckskil, Atlantic Ocean (Sweden)	FIS	Marine	58°25′N; 11°45′E	10	10	20
Mashinnoje (Russia)	MAS	Pond	66°18′N; 33°25′E	10	10	20
Pyöreälampi (Finland)	PYÖ	Pond	66°15′N; 29°26′E	10	10	20
Rytilampi (Finland)	RYT	Pond	66°23′N; 29°19′E	10	10	20
Abbortjärn (Sweden)	ABB	Pond	64°29′N; 19°26′E	10	10	20
Bynästjämen (Sweden)	BYN	Pond	64°27′N; 19°26′E	10	10	20
Total				110	70	180

ponds were extremely small (surface area < 5 ha; max. depth 5–10 m) stagnant water bodies, completely isolated and lacked predatory fish. The only sympatric fish species were three-spined sticklebacks (*Gasterosteus aculeatus*) in Mashinnoje (MAS) and small-sized whitefish (*Coregonus lavaretus*) in Pyöreälampi (PYÖ), both of which are potential competitors, but not predators of nine-spined sticklebacks. Although fish-eating birds were not observed around the ponds, occurrence of avian predation cannot be completely ruled out. However, it is not expected that the lateral line system would be involved in the detection of non-aquatic predators. The marine sampling sites were shallow coastal bays. In contrast to ponds, marine environments represent complex habitats with higher structural heterogeneity and higher potential hydrodynamic complexity (e.g. tides, currents, inflowing creeks), in addition to diverse fish communities composed of a large number of potential competitors and predators.

The populations included in this study have been shown to be genetically isolated from each other, as revealed by highly polymorphic microsatellite markers (Shikano *et al.*, 2010). Exceptions are the two Baltic Sea populations, Helsinki (HEL) and Bölesviken (BÖL), and the two Russian populations, Levin Navolok (LEV) and Mashinnoje (MAS), among which levels of neutral marker differentiation are low (Shikano *et al.*, 2010). This may be explainable by the lack of barriers against gene flow in the Baltic Sea, and recent isolation of the Russian ponds (within a century) from the White Sea (Ziuganov & Zotin, 1995; information from the White Sea Biological Station, Russian Academy of Sciences).

Common garden experiment

Detailed procedures are given elsewhere (see 'shoal treatment' in Herczeg *et al.*, 2009c). In short, adult fish were caught in early spring from two marine (HEL, Baltic Sea; LEV, White Sea) and two freshwater pond (BYN and PYÖ) populations (Fig. 1). Fish were transferred to aquaculture facilities at the University of Helsinki and reared in freshwater. Within each sampled population, five full-sib crosses were produced *in vitro*. Four days after hatching, each family was divided into two replicates. After ca. 7 weeks, 20 fish per family were randomly chosen (replicates equally represented) and pooled within population (n = 100 fish per population; except HEL, n = 93). At 1 year of age, all fish were euthanized, and a random sample of 20 individuals from each population (family origin unknown) was selected for use in the present study.

Neuromast counting

Both wild-caught and common garden fish were overanaesthetized with MS 222 (tricaine methanesulfonate) and stored in 96% ethanol. Samples were later fixed in 4% formalin. Standard staining procedure was used for visualization of neuromasts: samples were briefly dehydrated in 70% ethanol and placed in 1 g L^{-1} alizarin red; 0.5% KOH for 3 days. Fish were destained in 1% KOH for 4 days and transferred to alcohol. Superficial neuromasts and canal neuromast pores on the left lateral surface were counted under a dissecting microscope (Wild M5A; Wild, Heerbrugg, Switzerland). Neuromasts belonging to 17 different lateral line regions, six composed of canal neuromasts and 11 composed of superficial neuromasts (Fig. 2), were counted for 180 wild-caught and 80 laboratory-reared individuals. Additionally, neuromast counting was repeated a second time for a random sample of 20 individuals to estimate count repeatability. Repeatability (R) of all neuromast counts used in the analyses was high (mean R = 0.97, median R = 1, $P < 0.001$ in all tests).

Defining neutral genetic divergence

To assess the degree of neutral genetic differentiation among populations, 40 individuals from each of the populations were genotyped for 23 microsatellite loci. DNA was extracted from proteinase K digested fin clips with a phenol-chloroform (Taggart *et al.*, 1992) or a

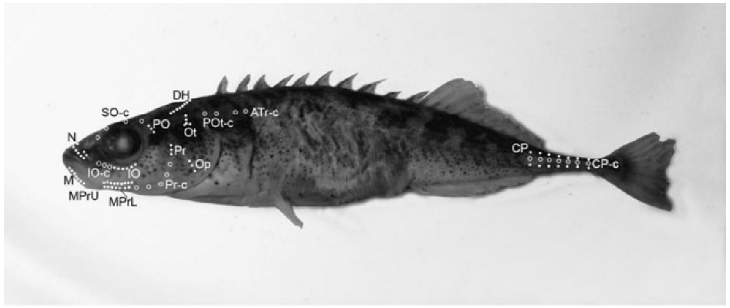


Fig. 2 Schematic presentation of the location of the lateral line structures used in this study. Superficial neuromasts are indicated as white dots and include the following corporal regions: nasal (N), mandibular (M), infraorbital (IO), upper mandibular preopercular (MPrU), lower mandibular preopercular (MPrL), post-orbital (PO), pre-opercular (Pr), dorsal head (DH), otic (Ot), operculum (Op), and caudal pedunde (CP). Canal neuromasts are indicated with a '-c' suffix and are shown as open circles. They include: supraorbital (SO-c), infraorbital (IO-c), pre-opercular (Pr-c), post-otic (POt-c), anterior trunk (ATr-c), and caudal peduncle (CP-c).

silica-fine-based purification method (Elphinstone *et al.* 2003). The following 23 microsatellite loci were genotyped: Gac1125PBBE, Gac4174PBBE, Gac7033PBBE, GAest3, GAest7, GAest14, GAest35, GAest50, GAest66, GAest82, Stn49, Stn71, Stn89, Stn96, Stn100, Stn127, Stn130, Stn163, Stn173, Stn196, Stn198, Stn223 and Stn253 (Largiadèr *et al.*, 1999; Peichel *et al.*, 2001; Colosimo *et al.*, 2004; Mäkinen *et al.*, 2008; Shikano *et al.*, 2010). Forward primers were labelled with fluorescent dyes (FAM, HEX or TET) for visualization of PCR products; the 5′-ends of the reverse primers were modified with an additional GTTT-tail to enhance the 3′-adenylation (Brownstein *et al.*, 1996). Loci with non-overlapping allele sizes were arranged in multiplex PCR panels, and all amplifications were carried out using the Qiagen Multiplex PCR Kit (Qiagen GmbH, Hilden, Germany) containing 1× Qiagen Multiplex PCR Master Mix, 0.5× Q-Solution, 2 ρmol of each primer, 10–20 ng of template DNA and MQ water for a final reaction volume of 10 μL. PCRs were performed using the following cycle: an initial denaturation step at 95 °C for 15 min, followed by 30 s at 94 °C, 90 s at 53 °C and 60 s at 72 °C for 30 cycles with a final extension at 60 °C for 5 min. PCR products were run on a MegaBACE 1000 automated sequencer (Amersham Biosciences), and their sizes determined against an ET-ROX 400 or 550 size standard (Amersham Biosciences). Alleles were scored with Fragment Profiler 1.2 (GE Healthcare Bio-Science Corp, Piscataway, NJ, USA) with visual inspection and manual correction.

Statistical methods

All analyses were performed using the R computing language (R Development Core Team, 2007). As the variables of interest (number of neuromasts per lateral line region) represent count data, assumptions of normality and sphericity underlying traditional multivariate techniques were violated. Consequently, to assess differences between pond and marine fish, a non-parametric multivariate analysis of variance (MANOVA; Anderson, 2001; McArdle & Anderson, 2001) was used. Significance tests were performed via permutation (1000 randomizations), employing strata to account for nesting, in this case sampled populations nested within each environment. Quantification of dispersion in multivariate space was accomplished by calculating the Euclidean distance between each individual and its group centroid; heterogeneity of variance was evaluated by ANOVA of these metrics. Analyses employed the functions implemented in the 'vegan' package (Oksanen *et al.*, 2007).

Data visualization and evaluation of the relative contributions of each variable (component lateral line regions) to discriminate between environments were performed using between-class ordination (ter Braak, 1986; Holmes, 2008). Between-class correspondence analysis, and eigenanalyses of component variables were performed using the 'ade4' package (Dray & Dufour, 2007). Additionally, we complemented multivariate analyses with univariate analyses of each lateral line region. These data were evaluated within the framework of a Bayesian generalized linear mixed model (GLMM), implemented in the 'MCMCglmm' package (Hadfield, 2010). Count data were modelled with a log link function and assuming Poisson distributed errors. Each model consisted of a 'burnin' of 100 000 runs, followed by 1 000 000 Markov chain Monte Carlo simulations from which each 1000th point was sampled to minimize autocorrelation of parameter estimates. Fixed factors included the effects of habitat (marine vs. pond), sex

and their interaction. Variation among populations was included as a random effect. Significance of fixed effects was evaluated by sampling the posterior distribution of parameter estimates. Random effects were evaluated by comparison of the deviance information criterion (DIC) of the full model against a simpler model excluding random effects (Spiegelhalter *et al.*, 2002).

To characterize lateral line divergence among samples, the index of phenotypic divergence (P_{ST}; eqn 1), a surrogate of the quantitative genetic divergence index (Q_{ST}; e.g. Spitze, 1993) estimated from phenotypic data alone (Leinonen *et al.*, 2006; Pujol *et al.*, 2008), was calculated for or each lateral line region as:

$$P_{ST} = \frac{\sigma^2_{PB}}{\sigma^2_{PB} + 2\sigma^2_{PW}} \quad (1)$$

where σ^2_{PB} denotes phenotypic variance between/among, and σ^2_{PW} that within groups. For this purpose, data were square root transformed, and phenotypic variance between/among (σ^2_{PB}) and within groups (σ^2_{PW}) was estimated by standard variance components analysis. Confidence intervals of estimates were obtained by non-parametric bootstrapping (5000 iterations). Three resultant estimates corresponding to (i) divergence between environments (marine vs. pond), (ii) divergence among populations within the marine environment, and (iii) divergence among populations within the pond environment were compared to an index of putatively neutral differentiation based on average genetic differences (F_{ST}) between/among groups calculated from 23 informative microsatellite loci. Confidence limits for F_{ST} were estimated from 10 000 bootstrap iterations using the 'hierfstat' package (Goudet, 2005). Additionally, we complemented these analyses by testing for a positive correlation between pairwise (between sites) matrices of P_{ST} and habitat similarity (a dummy variable wherein '0' denotes sites from the same habitat type, and '1' denotes sites of differing habitat type), independent of co-variation with F_{ST} (Saether *et al.*, 2007). The R package 'vegan' was used to perform partial Mantel tests of matrix correlations, with significance evaluated by 10 000 permutations (Oksanen *et al.*, 2007).

Results

Habitat-based divergence

Nested MANOVA indicated a clear and significant (Table 2, perm. $P = 0.009$) difference in overall neuromast count between sticklebacks from pond and marine habitats, even after accounting for significant-among population variation within each environment. Three canonical axes accounted for 77.8% of variation in neuromast number (Table S1). Results of between-class correspondence analysis revealed that anterior trunk canal neuromast (ATr-c; Fig. 2) and caudal peduncle neuromast numbers (superficial and canal, CP and CP-c; Fig. 2) were the traits contributing most to between-habitat differentiation, as defined by the first canonical axis (Fig. 3b,c; Table S1). Univariate analyses of all traits were also consistent with these findings: only the number of anterior trunk canal neuromasts (ATr-c) and caudal peduncle neuromasts (CP and CP-c) differed significantly between habitats (Table 3; Fig. S1). Eleven of the 17 traits also showed significant among-population variation in univariate analyses (Table 3).

Table 2 Nested nonparametric MANOVA results. Differences in multivariate neuromast count data for each lateral line region are evaluated between main groups, in addition to average variation nested within each group, indicated in square brackets. Analyses of wild-caught fish include a contrast between fish from marine and pond environments (habitat), or between sexes within each habitat type. Comparison of data from wild-caught and laboratory-reared fish correspond to only a subset of populations used in this study (see Materials and methods). These analyses contrast fish originating from different habitats, in addition to differences between laboratory and wild fish within each habitat group. Mean square (MS), sum of square (SS).

Source of variance	d.f.	SS	MS	*F*	R^2	*P*	Source of variance	d.f.	SS	MS	*F*	R^2	*P*
Wild-caught fish							Comparison of wild-caught and laboratory-reared fish						
Habitat	1	0.539	0.539	3.238	0.227	0.009	Habitat	1	0.782	0.782	5.464	0.347	0.032
[Population]	7	1.165	0.166	42.777	0.492	0.001	[Population/treatment]	6	0.859	0.143	35.605	0.381	0.001
Residual	171	0.665	0.004				Residual	152	0.611	0.004			
Total	179	2.369					Total	159	2.252				
Pond fish only													
Sex	1	0.043	0.043	0.329	0.029	0.693	Group (laboratory vs. wild)	1	0.077	0.077	0.242	0.072	1.000
[Population]	8	1.044	0.131	29.764	0.705	0.001	[Population]	2	0.635	0.318	66.949	0.592	0.001
Residual	90	0.395	0.004				Residual	76	0.361	0.005			
Total	99	1.482					Total	79	1.073				
Marine fish only													
Sex	1	0.069	0.069	2.937	0.198	0.105	Group (laboratory vs. wild)	1	0.015	0.015	0.231	0.038	0.670
[Population]	4	0.094	0.023	9.375	0.270	0.001	[Population]	2	0.131	0.066	19.945	0.331	0.001
Residual	74	0.185	0.003				Residual	76	0.250	0.003			
Total	79	0.348					Total	79	0.397				

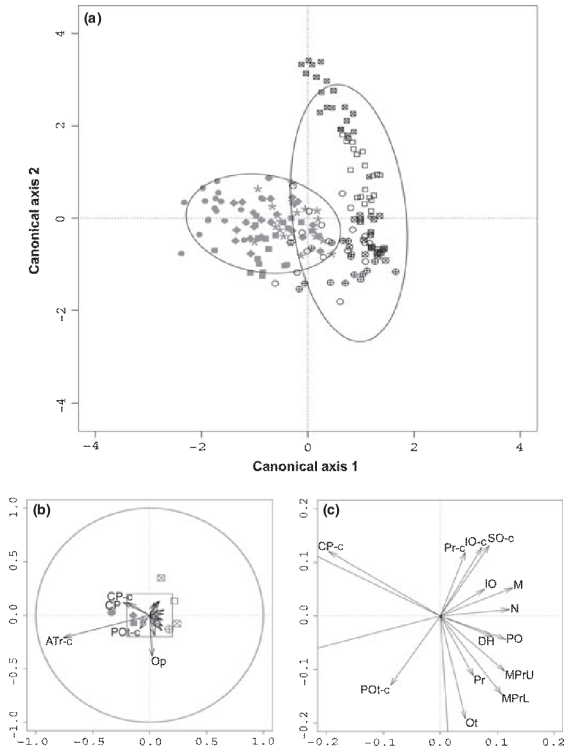


Fig. 3 Between-group correspondence analysis of wild-caught fish. (a) Individual scores for the first two canonical axes. Symbols for the population of origin correspond to those in Fig. 1, with marine fish plotted in grey, and open black symbols denoting fish from ponds. 95% confidence ellipses for each group are shown. (b) Correlation circle of vector loadings. Symbols denote the centroid of each respective population (as in Fig. 1). The region outlined in the centre (black square) is magnified in (c).

Multivariate heterogeneity of variance

In addition to describing variation between habitat types, the first canonical axis also appeared to capture among population variation within the marine environment, whereas the second canonical axis captured substantial variation among pond populations (Fig. 3a). Tests for homogeneity of multivariate dispersion revealed significant differences among populations from different habitats (perm. $P < 0.001$). Analyses of average within-group distance from their respective centroids indicated greater overall heterogeneity within pond populations compared to marine populations (Fig. 4). Similar patterns were also observed in the dispersion of most neuromast locations when plotted individually (12/15 variable traits; Fig. S1).

Absence of sexual dimorphism

No difference was detected between sexes, independent of between-habitat variation (perm. $P = 0.654$), nor were there differences between sexes in either environment, independent of among-population variation (Table 2). Univariate analyses were largely concordant with multivariate results: we detected no significant interaction between habitat and sex (H × S) for any lateral line region, although one (ATr-c) exhibited a significant effect of sex, averaged over habitat types (Table 3).

Comparison of laboratory-reared and wild-caught fish

Analysis of first-generation offspring originating from a subset of marine and pond populations reared under

Table 3 Habitat-based differentiation in neuromasts (see also Fig. S1). Univariate analyses of neuromast numbers for 17 lateral line regions are based on Bayesian generalized mixed-effects model with Poisson distributed error and a log-link function. Fixed factors include the effects of habitat (pond vs. marine), sex and their interaction (H × S). Significance of fixed effects (*P*) was evaluated with Markov chain Monte Carlo sampling of the posterior distribution for each coefficient, conditional upon random variation among populations. Var Estimates = Estimates for random variation among populations within habitat (Pop) and residual (resid) model variance. DIC = deviance information criterion contrasting a simpler model based only on fixed effects with one incorporating random effects. Smaller values of DIC are indicative of the preferred (parsimonious) model. Values in bold indicate 'significant' random effects. A significant (based on 10 000 permutations), positive correlation (*r*) between matrices of P_{ST} and habitat distance, independent of co-variation with F_{ST}, is indicative of divergent selection between pond and marine habitats. Note that partial correlations could not be calculated (n.c.) for neuromasts with no variation.

	Fixed effects *P*			Var. Estimates		Model DIC		
Neuromast	Habitat	Sex	H × S	Pop.	Resid.	Fixed	Random	Mantel *r* (*P*)
Nasal	0.622	0.944	0.520	0.025	0.000	778.20	**768.46**	–0.424 (0.739)
Mandibular	0.248	0.376	0.850	0.004	0.001	695.46	697.45	0.036 (0.422)
Infraorbital	0.530	0.764	0.454	0.002	0.000	698.23	701.47	–0.067 (0.663)
Upper mandibular preopercular	0.738	0.354	0.708	0.099	0.001	742.80	**701.00**	0.090 (0.415)
Lower mandibular preopercular	0.700	0.652	0.692	0.137	0.001	696.90	**657.02**	–0.029 (0.503)
Post-orbital	0.560	0.676	0.978	0.022	0.001	549.18	549.50	–0.153 (0.546)
Pre-opercular	0.304	0.716	0.654	0.101	0.001	565.81	**549.35**	0.805 (0.164)
Dorsal head	0.838	0.714	0.358	0.048	0.001	712.51	**689.27**	–0.785 (1.000)
Otic	0.394	0.914	0.436	0.237	0.001	704.43	**640.59**	–0.496 (0.706)
Operculum	0.538	0.556	0.938	1.814	0.002	612.03	**496.21**	–0.350 (0.585)
Caudal peduncle	< 0.001	0.084	0.374	0.043	0.001	766.14	**742.88**	0.136 (0.500)
SO-c	0.986	0.968	0.866	0.001	0.001	602.87	605.30	n.c.
IO-c	0.968	0.878	0.886	0.001	0.001	595.55	598.04	n.c.
Pr-c	0.612	0.992	0.872	0.002	0.001	639.22	640.71	0.553 (0.249)
POt-c	0.292	0.974	0.836	0.336	0.001	500.36	**472.87**	0.471 (0.163)
ATr-c	0.040	0.016	0.476	8.446	0.002	642.78	**482.10**	0.964 (0.044)
CP-c	< 0.001	0.144	0.424	0.037	0.000	781.98	**762.78**	0.136 (0.500)

Canal neuromasts are indicated with a '-c' suffix. They include: (SO-c), infraorbital (IO-c), pre-opercular (Pr-c), post-otic (POt-c), anterior trunk (ATr-c), and caudal peduncle (CP-c).

common/controlled environmental conditions was consistent with the results from wild-caught samples. The first three canonical axes of the multi-group correspondence analysis captured 79.6% of total variation among samples (Table S1). As in the analysis of wild data, the first axis defined differences between samples from pond and marine habitats, with anterior trunk canal neuromast and caudal peduncle (superficial and canal) neuromast variation contributing most significantly to observed differences (Table S1). The second axis was defined principally by variation in opercular neuromast numbers, and described variation among samples, predominantly among pond groups (Table S1). The third axis held no biologically evident information.

In general, ordination plots of laboratory-reared fish and individuals sampled from corresponding source populations showed significant overlap (Fig. 5). This was most evident in marine samples that exhibited relatively little centroid variation among groups (Fig. 5). More variation was observed among pond samples, primarily because of wild-caught samples from Bynästjärnen (Fig. 5). Nevertheless, despite variation among samples, we continued to detect significant differences between sticklebacks from different habitat types (perm. $P = 0.032$; Table 2), whereas contrasts within each environment revealed no significant differences between wild-caught and laboratory-reared fish (Table 2). This within-habitat group similarity in neuromast number between wild-caught and laboratory-reared fish – as well as between-habitat differences – was also evident in univariate data (Fig. S2).

Phenotypic vs. neutral divergence

For the majority of lateral line regions, the degree of divergence in neuromast numbers overlapped with the range of neutral expectation, defined by variation in microsatellite markers (Fig. 6). Contrasting neuromast variation between pond and marine habitats revealed three lateral line regions (ATR-c, CP and CP-c) for which levels of divergence exceeded neutral expectations (Fig. 6a). This result was corroborated by partial Mantel tests: only ATR-c, CP and CP-c exhibited significant positive correlations between matrices of P_{ST} and habitat distance, independent of co-variation with F_{ST} (Table 3; Fig. S3). These lateral line regions also exhibited similar patterns of divergence among populations within the marine environment (Fig. 6c), but not among pond populations (Fig. 6b). Only two lateral line regions (SO-c and IO-c) exhibited consistent variation below neutral expectation in all comparisons.

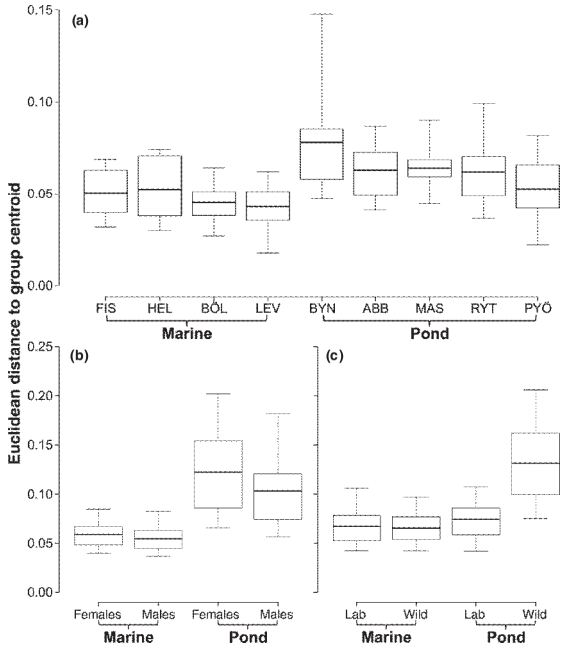


Fig. 4 Multivariate heterogeneity of variance, as indicated by average individual distance from their group centroid. Box plots denote the interquartile range, whereas whiskers denote 95% quantiles. (a) Wild-caught individuals compared to their population-specific centroid. (b) Sex-specific distances to the overall centroids for marine and pond groupings. (c) Comparison of laboratory-reared and wild-caught fish from the marine and pond environments.

Discussion

Our results demonstrated marked intraspecific variation and differentiation in the lateral line system of nine-spined sticklebacks. Conversely, there was no evidence to indicate sexual dimorphism in neuromast number, suggesting that habitat-specific differences are far more important determinants of neuromast counts than any sex-related factors. This inference is further strengthened by the fact that habitat-based divergence was detected independent of any variation among populations within each habitat. Because repeatedly and independently derived occurrence of the same phenotype in populations inhabiting similar habitats strongly implies natural selection as the causal agent (Clarke, 1975; Endler, 1986; Schluter & Nagel, 1995; Foster, 1999; McGuigan *et al.*, 2005), the results suggest that the observed habitat-dependent patterns might reflect evolutionary adaptation to different biotic and/or abiotic environmental conditions faced by nine-spined sticklebacks in marine and pond environments. Moreover, the divergent patterns of neuromast variability within habitats (pond > marine) may be equally revealing into the nature of selective differences between these habitats and the evolutionary history of the stickleback populations inhabiting them. In what follows, we will discuss these findings and their implications for our understanding of local adaptation and neuromast variability.

A genetic basis to neuromast variation?

A critical assumption in any study wishing to make evolutionary inference on the basis of data collected from the wild is that the observed phenotypic patterns are not environmentally induced, but reflect genetically based differences (e.g. Conover & Schultz, 1995). To probe the degree to which observed habitat-based differences in this study reflected genetic variability (cf. local adaptation) and/or phenotypic plasticity, we preformed a simple common garden experiment. By rearing fish under controlled laboratory conditions, wherein environmental factors that might influence lateral line development were homogeneous, any differences in neuromast number between groups originating from different environments should reflect genetically based differences. With the exception of Bynästjärnen individuals, which exhibited a greater amount of variation,

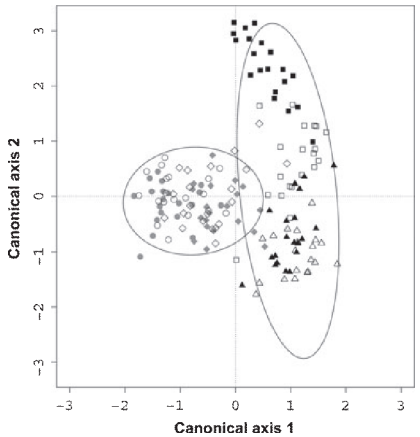


Fig. 5 Individual scores from between-class correspondence analysis of wild-caught and laboratory-reared fish. Closed symbols indicate wild-caught fish, whereas open symbols denote laboratory-reared individuals. Individuals of marine origin are plotted in grey: LEV (circles) and HEL (diamonds). Pond fish are plotted in black: BYN (squares) and PYÖ (triangles).

laboratory-reared and wild-sampled fish clustered/overlapped by population of origin (Fig. 5). More pertinent to the question of inferring a genetic basis to neuromast number, however, was the observation that comparisons of laboratory-reared and wild-caught fish revealed consistent and significant differences between habitat groups, even within the homogenous laboratory environment. This suggests that differences observed between habitat types in the wild are unlikely explained by phenotypic plasticity alone. Also, the fact that marine populations showed similar phenotypes in the wild (saline water) and in the laboratory (freshwater) argues against salinity-based phenotypic plasticity.

Although a genetic basis for observed differentiation is suggested by the facts reviewed above, this is not to say that environmental sources of phenotypic variation are unimportant within this system. That variation was reduced when fish were reared under controlled conditions is a strong indicator of some degree of trait plasticity (Fig. 4b). Nevertheless, it is particularly interesting that the group (i.e. pond fish) that exhibited the greatest variation in the wild also exhibited the most substantial reduction of within-group variability when reared in the laboratory. This may be indicative of greater plasticity inherent in pond populations. Alternatively, the greater variation observed in wild pond fish, relative to that seen in the laboratory, may be attributable to more genetic diversity potentially sampled in the wild, compared to the limited number of few full-sib families used in the laboratory. However, this scenario seems unlikely, given the reduced genetic diversity of pond populations compared to marine ones (Shikano *et al.*, 2010). Another possible explanation would be higher selection pressure in the more complex/'dangerous' marine environment that could be expected reduce variance in neuromast counts. In fact, marine populations appeared relatively homogeneous as compared to pond populations (Fig. 4b,c). However, predation in the wild cannot explain reduced variability among laboratory-reared pond fish as compared with those from the wild. Hence, greater plasticity inherent in fish from pond populations remains a viable hypothesis to be investigated in further experiments.

Divergent selection between marine and pond environments

The three lateral line regions that showed significant habitat effects (CP, CP-c and ATr-c) were also the ones that were the most divergent in the marine environment. This suggests that these lateral line regions are of particular adaptive significance and that observed divergence between habitats is most likely the result of selection within the marine environment. This notion is further strengthened by the finding that phenotypic divergence in these traits clearly exceeded the neutral genetic baseline (Fig. 6a). Of course this interpretation comes with the major caveat that an index of trait divergence based strictly on phenotypic variance may confound environmentally based differences between contrasted groups with the action of selection (Pujol *et al.*, 2008). However, as outlined earlier, observed habitat-based differences are unlikely to be explainable by plasticity alone. Interestingly, divergence in these same three traits among pond populations conform to levels expected under drift (Fig. 6b), whereas patterns observed among marine samples are consistent with adaptive differentiation (Fig. 6c). Taken together, these observations and considerations suggest that the habitat divergence in neuromast counts in these three lateral line regions is driven by natural selection. Given the presumed functional significance of lateral line variation, we may hypothesize that the most likely cause for observed habitat-based trait divergence may be found from varying predation pressure.

Predation risk is an important difference between the marine and pond environments. Whereas study ponds are free of fish predators (Herczeg *et al.*, 2009a–c), the more complex marine communities contain numerous predatory species, and hence, senses involved in predator detection are likely to be under strong positive directional selection. Predation can influence fish behaviour in many ways, including induced schooling (e.g. Magurran, 1990). Importantly, neuromasts are known to mediate

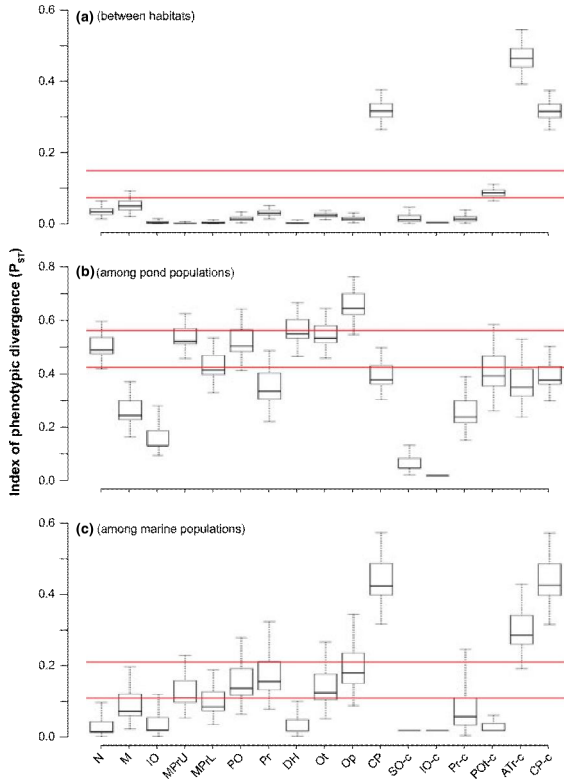


Fig. 6 Index of phenotypic divergence (P_{ST}) for different neuromast traits in (a) between habitat, (b) among pond population and (c) among marine population comparisons. Box plots denote the interquartile range and whiskers 95% quantiles of nonparametric bootstrap estimates (10 000 iterations). Horizontal lines denote the region of expected neutral divergence, indicated by upper and lower 95% confidence intervals of F_{ST} as estimated from microsatellites.

schooling (Pitcher *et al.*, 1976; Partridge & Pitcher, 1980). This, together with the fact that schooling can interfere with the lateral line sensory system of predators (Larsson, 2009), provides a potential cue to explain our observations. Namely, selection for increased and/or optimal neuromast numbers in the marine environment could explain the lower heterogeneity in neuromast counts in marine populations (where schooling is needed) as compared to pond populations (where schooling is not needed). In contrast, relaxed selection and genetic drift might explain the high within- (Fig. 4) and among (Fig. 6)-pond variance in neuromast counts. However, we cannot exclude other possible sources of natural selection contributing/resulting in the patterns reported: besides predation risk, salinity differs between marine and pond populations, as does the estimated level of aggression/intraspecific competition (Herczeg *et al.*, 2009b,c).

Between-habitat comparisons of lateral line divergence revealed that the majority of neuromasts had diverged less than expected by genetic drift alone (Fig. 6a). Although this could be indicative of potential trait canalization or stabilizing selection, we are hesitant to draw this conclusion given our inability to estimate the genetic components of trait variance, and the difficulty of interpreting patterns of $Q_{ST} < F_{ST}$ (Goudet & Büchi, 2006; Whitlock, 2008). Furthermore, among-population comparisons within each environment revealed that most estimates of trait divergence fell within the range expected under neutrality, thus indicating a high likeli-

hood of trait drift. However, pond populations showed greater overall heterogeneity in lateral line neuromast numbers compared to marine populations, with values of P_{ST} (and F_{ST}) being in general much higher for the pond populations. From this, it is tempting to suggest that drift may be more prominent within the pond environment, but this too may be complicated by the greater degree of neutral genetic divergence observed among pond populations (Fig. 6b). In marine populations, there is a greater likelihood for gene flow which could be keeping F_{ST} comparatively low. Alternatively, differences in F_{ST} might be more reflective of greater effective population size (N_e) within the marine environment, in which case the naturally smaller/constrained ponds again should be more influenced by random processes (Wright, 1931; Holsinger & Weir, 2009). Taken together, this is suggestive of a greater potential for drift in ponds and 'canalization' in the marine environment. However, this is only weak inference, albeit an interesting one worthy of comment and future research.

Finally, it should be noted that some interspecific studies have reported differing associations among canal vs. superficial lateral line neuromast numbers and environmental factors, particularly in relation to predation differences (e.g. Webb, 1989; Montgomery *et al.*, 1995). Our data were consistent with such expectations of greater canal neuromast divergence between environments differing in predation regimes, given that two of the three most divergent lateral line regions were canal neuromasts (ATr-C and CP-c) and that marine fish had significantly more of these neuromasts (Table 3; Fig. S1). Moreover, a rudimentary test of this hypothesis found some evidence to suggest that canal neuromasts may be more divergent between pond and marine environments than superficial neuromasts: between-habitat MANOVA results were significant for analyses performed on canal neuromasts alone (perm. $P = 0.008$), whereas superficial neuromast numbers did not differ (perm. $P = 0.406$). However, these results must interpreted cautiously given that there are fewer lateral line regions composed of canal neuromasts ($n = 6$) than superficial neuromasts ($n = 17$), and it is unknown to what extent neuromast-dependent differences in habitat discrimination are influenced by this 'unbalanced' comparison.

Conclusions

Taken together, we found significant divergence in the lateral line system of nine-spined sticklebacks between pond and marine habitats. Results from a common garden experiment indicated that this divergence has a genetic component, and results of $P_{ST} - F_{ST}$ comparisons further suggested that the habitat-based differences of three neuromast traits are likely to have been caused by natural selection. Adaptive differentiation of this kind between the two habitats is not unexpected given that the mechanosensory lateral line system is an important organ system conveying information crucial to individual fitness, and earlier studies have found that the nine-spine sticklebacks from these two these two habitat types have diverged also in their morphology (Herczeg *et al.*, 2009a, 2010a,b), behaviour (Herczeg *et al.*, 2009b,c) and neural architecture (Gonda *et al.*, 2009a,b). Although the latter studies suggest that much of the differentiation between marine and pond habitats is ascribable to predation-mediated selection (or its relaxation), a more detailed understanding of the function, and link to fitness, of neuromast variation awaits further investigation. To this end, we hope that the findings of this study inspire further intraspecific research on mechanosensory lateral system variability and function in this and other model systems.

Acknowledgments

We thank Victor Berger, Jacquelin DeFaveri, Göran Englund, Abigél Gonda, Lotta Kvarnemo, Tuomas Leinonen, Daniel Lussetti, Jouko Pokela and Pirkko Siikamäki for their help in obtaining the samples. We are indebted to the Oulanka Research Station (University of Oulu) and the White Sea Biological Station (Russian Academy of Sciences) for their help with field logistics. Thanks are also due to Jacquelin DeFaveri for comments on an earlier version of the manuscript. Our research was funded by Ella and Georg Ehrnrooth Foundation (N.T.), Academy of Finland (G.H, J.M; grant nos 129662, 134728) and The Finnish Ministry of Education (J.M.). Parts of the work were conducted within the framework of the BaltGene project funded by BONUS Baltic Organisations' Network for Funding Science EEIG (J.M.). The experiment was conducted under the licence of the Helsinki University Animal Experimentation Committee (STH379A).

References

Anderson, M.J. 2001. A new method for non-parametric multivariate analysis of variance. *Austral. Ecol.* **26**: 32–46.

Bănărescu, P.M. & Paepke, H.J. 2001. *The Freshwater Fishes of Europe*, Vol 5/III. AULA-Verlag, Wiebelsheim.

Blaxter, J.H.S. & Fuiman, L.A. 1990. The role of the sensory systems of herring larvae in evading predatory fishes. *J. Mar. Biol.* **70**: 413–427.

ter Braak, C.J.F. 1986. Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. *Ecology* **67**: 1167–1179.

Brownstein, M.J., Carpten, J.D. & Smith, J.R. 1996. Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. *BioTechniques* **20**: 1004–1010.

Cahn, P.H., Shaw, E. & Atz, E.H. 1968. Lateral line histology as related to the development of schooling in the atherinid fish. *Menidia. Bull. Mar. Sci.* **18**: 660–670.

Campenhausen, C., Riess, I. & Weissert, R. 1981. Detection of stationary objects by the blind cave fish *Anoptichthys jordani* (Characidae). *J. Comp. Physiol.* **143**: 369–374.

Carton, A.G. & Montgomery, J.C. 2004. A comparison of lateral line morphology of blue cod and torrentfish: two sandperches of the family Pinguipedidae. *Environ. Biol. Fishes* **70**: 123–131.

Clarke, B.C. 1975. The contribution of ecological genetics to evolutionary theory: detecting the direct effects of natural selection on particularly polymorphic loci. *Genetics* **79**: 101–113.

Colosimo, P.F., Hosemann, K.E., Balabhadra, S. *et al.* 2005. Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. *Science* **307**: 1928–1933.

Conover, D.O. & Schultz, E.T. 1995. Phenotypic similarity and the evolutionary significance of countergradient variation. *Trends Ecol. Evol.* **10**: 248–252.

Coombs, S., Janssen, J. & Webb, J.F. 1988. Diversity of lateral line systems, evolutionary and functional considerations. In: *Sensory Biology of Aquatic Animals* (J. Atema, R.R. Fay, A.N. Popper & W.N. Tavolga, eds), pp. 553–593. Springer-Verlag, Heidelberg.

Coombs, S., Finneran, J. & Conley, R.A. 2000. Hydrodynamic imaging by the lateral line system of the Lake Michigan mottled sculpin. *Phil. Trans. Roy. Soc. Lond.* **355**: 1111–1114.

Coombs, S., Braun, C.B. & Donovan, B. 2001. Orienting response of Lake Michigan mottled sculpin is mediated by canal neuromasts. *J. Exp. Biol.* **204**: 337–348.

Dangles, O., Magal, C., Pierre, D., Olivier, A. & Casas, J. 2005. Variation in morphology and performance of predator-sensing system in wild cricket populations. *J. Exp. Biol.* **208**: 461–468.

Dray, S. & Dufour, A.B. 2007. The ade4 package: implementing the duality diagram for ecologists. *J. Stat. Software* **22**: 1–20.

Endler, J. 1977. *Geographic Variation, Speciation & Clines.* Princeton University Press, Princeton, NJ.

Endler, J.A. 1986. *Natural Selection in the Wild.* Princeton University Press, Princeton, NJ.

Endler, J.A., Basolo, A.L., Glowacki, S. & Zerr, J. 2001. Variation in response to artificial selection for light sensitivity in guppies (*Poecilia reticulata*). *Am. Nat.* **158**: 36–48.

Engelmann, J., Hanke, W., Mogdans, J. & Bleckmann, H. 2000. Hydrodynamic stimuli and the fish lateral line. *Nature* **408**: 51–52.

Elphinstone, M.S., Hinten, G.N., Anderson, M.J. & Nock, C.J. 2003. An inexpensive and high-throughput procedure to extract and purify total genomic DNA for population studies. *Mol. Ecol. Notes* **3**: 317–320.

Foster, S.A. 1999. The geography of behaviour: an evolutionary perspective. *Trends Ecol. Evol.* **14**: 190–195.

Fuiman, L.A. 1993. Development of predator evasion in Atlantic herring, *Clupea harengus* L. *Anim. Behav.* **45**: 1101–1116.

Fuller, R.C., Fleishman, L.J., Leal, M., Travis, J. & Loew, E. 2003. Intraspecific variation in retinal cone distribution in the bluefin killifish, *Lucania goodei*. *J. Comp. Physiol.* **189**: 609–616.

Gallego, A. & Heath, M.R. 1994. The development of schooling behaviour in Atlantic herring *Clupea harengus*. *J. Fish Biol.* **45**: 569–588.

Gonda, A., Herczeg, G. & Merilä, J. 2009a. Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain. *Proc. R. Soc. B* **276**: 2085–2092.

Gonda, A., Herczeg, G. & Merilä, J. 2009b. Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? *J. Evol. Biol.* **22**: 1721–1726.

Goudet, J. 2005. HIERFSTAT, a package for R to compute and test hierarchical *F*-statistics. *Mol. Ecol. Notes* **5**: 184–186.

Goudet, J. & Büchi, L. 2006. The effects of dominance, regular inbreeding and sampling design on Q_{ST}, an estimator of population differentiation for quantitative traits. *Genetics* **172**: 1337–1347.

Guillén, A., Juste, B.J. & Ibáñez, C. 2000. Variation in the frequency of the echolocation calls of *Hipposideros ruber* in the Gulf of Guinea: an exploration of the adaptive meaning of the constant frequency value in rhinolophoid CF bats. *J. Evol. Biol.* **13**: 70–80.

Hadfield, J.D. 2010. MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package. *J. Stat. Software* **33**: 1–22.

Herczeg, G., Gonda, A. & Merilä, J. 2009a. Evolution of gigantism in nine-spined sticklebacks. *Evolution* **63**: 3190–3200.

Herczeg, G., Gonda, A. & Merilä, J. 2009b. Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *J. Evol. Biol.* **22**: 544–552.

Herczeg, G., Gonda, A. & Merilä, J. 2009c. The social cost of shoaling covaries with predation risk in ninespine stickleback, *Pungitius pungitius*, populations. *Anim. Behav.* **77**: 575–580.

Herczeg, G., Gonda, A. & Merilä, J. 2010a. Rensch's rule inverted – female-driven gigantism in nine-spined stickleback *Pungitius pungitius*. *J. Anim. Ecol.* **79**: 581–588.

Herczeg, G., Turtiainen, M. & Merilä, J. 2010b. Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. *Biol. J. Linn. Soc.* **101**: 403–416.

Hoekstra, D. & Janssen, J. 1985. Non-visual feeding behavior of the mottled sculpin, *Cottus bairdi*, in Lake Michigan. *Env. Biol. Fish.* **12**: 111–117.

Holmes, S. 2008. Multivariate data analysis: the French way. In: *Probability and Statistics: Essays in Honor of David A. Freedman* (D. Nolan & T. Speed, eds), pp. 219–233. Institute of Mathematical Statistics, Beachwood, OH.

Holsinger, K.E. & Weir, B.S. 2009. Genetics in geographically structured populations: defining, estimating and interpreting F_{ST}. *Nat. Rev. Genet.* **10**: 639–650.

Honkanen, T. 1993. Comparative study of the lateral-line system of the 3-spined stickleback (*Gasterosteus aculeatus*) and the 9-spined stickleback (*Pungitius pungitius*). *Acta Zool.* **74**: 331–336.

Kalmijn, A.J. 1988. Hydrodynamic and acoustic field detection. In: *Sensory Biology of Aquatic Animals* (J. Atema, R.R. Fay, A.N. Popper & W.N. Tavolga, eds), pp. 83–130. Springer-Verlag, Heidelberg.

Largiadèr, C.R., Fries, V., Kobler, B. & Bakker, T.C. 1999. Isolation and characterization of microsatellite loci from the threespined stickleback (*Gasterosteus aculeatus* L.). *Mol. Ecol.* **8**: 342–344.

Larsson, M. 2009. Possible functions of the octavolateralis system in fish schooling. *Fish Fish.* **10**: 344–353.

Leinonen, T., Cano Arias, J.M., Mäkinen, H. & Merilä, J. 2006. Contrasting patterns of body shape and neutral genetic divergence in marine and lake populations of threespined sticklebacks. *J. Evol. Biol.* **19**: 1803–1812.

Magurran, A.E. 1990. The adaptive significance of schooling as an anti-predator defence in fish. *Ann. Zool. Fennici* **27**: 51–66.

Mäkinen, H.S. & Merilä, J. 2008. Mitochondrial DNA phylogeography of the three-spined stickleback (*Gasterosteus aculeatus*) in Europe – evidence for multiple glacial refugia. *Mol. Phylogenet. Evol.* **46**: 167–182.

Maruska, K.P. 2001. Morphology of the mechanosensory lateral line system in elasmobranch fishes: ecological and behavioral considerations. *Environ. Biol. Fish* **60**: 47–75.
Mayr, E. 1963. *Animal Species and Evolution*. Harvard University Press, Cambridge.
McArdle, B.H. & Anderson, M.J. 2001. Fitting multivariate models to community data: a comment on distance-based redundancy analysis. *Ecology* **82**: 290–297.
McClelland, B.E., Wilczynski, W. & Ryan, M.J. 1998. Intraspecific variation in laryngeal and ear morphology in male cricket frogs (*Acris crepitans*). *Biol. J. Linn. Soc.* **63**: 51–67.
McGuigan, K., Chenoweth, S.F. & Blows, M.W. 2005. Phenotypic divergence along lines of genetic variance. *Am. Nat.* **165**: 32–43.
Michel, C., Hicks, B.J., Stölting, K.N., Clarke, A.C., Stevens, M.I., Tana, R. *et al*. 2008. Distinct migratory and non-migratory ecotypes of an endemic New Zealand eleotrid (*Gobiomorphus cotidianus*) – implications for incipient speciation in island freshwater fish species. *BMC Evol. Biol.* **8**: 1–14.
Montgomery, J.C. & Macdonald, J.A. 1987. Sensory tuning of lateral line receptors in Antarctic fish to the movements of planktonic prey. *Science* **235**: 195–196.
Montgomery, J.C. & Milton, R.C. 1993. Use of the lateral line for feeding in the torrent fish (*Cheimarrichthys fosteri*). *New Zealand J. Zool.* **20**: 121–125.
Montgomery, J., Coombs, S. & Halstead, M. 1995. Biology of the mechanosensory lateral line in fishes. *Rev. Fish Biol. Fish.* **5**: 399–416.
Montgomery, J.C., Baker, C.F. & Carton, A.G. 1997. The lateral line can mediate rheotaxis in fish. *Nature* **389**: 960–963.
Montgomery, J.C., Coombs, S. & Baker, C.F. 2001. The mechanosensory lateral line system of the hypogean form of *Astyanax fasciatus*. *Environ. Biol. Fish* **62**: 87–96.
Oksanen, J., Kindt, R., Legendre, P. & O'Hara, R.B. 2007. *Vegan: Community Ecology Package. R package Version 1.8–6*. http://ocw.um.es/ciencias/geobotanica/otros-recursos-1/documentos/vegan.pdf.
Partridge, B.L. & Pitcher, T.J. 1980. The sensory basis of fish schools, relative roles of lateral line and vision. *J. Comp. Physiol.* **135**: 315–325.
Peichel, C.L., Nereng, K.S., Ohgi, K.A. *et al*. 2001. The genetic architecture of divergence between threespine stickleback species. *Nature* **414**: 901–905.
Pitcher, T.J., Partridge, B.L. & Wardle, C.S. 1976. A blind fish can school. *Science* **194**: 963–965.
Pujol, B., Wilson, A.J., Ross, R.I.C. & Pannell, J.R. 2008. Are $Q_{ST}-F_{ST}$ comparisons for natural populations meaningful? *Mol. Ecol.* **17**: 4782–4785.
R Development Core Team 2007. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
Saether, S.A., Fiske, P., Kålås, J.A., Kuresoo, A., Luigujõe, L., Piertney, S.B. *et al*. 2007. Inferring local adaptation form $Q_{ST}-F_{ST}$ comparisons: neutral genetic and quantitative trait variation in European populations of the great snipe. *J. Evol. Biol.* **20**: 1563–1576.
Saunders, A.J. & Montgomery, J.C. 1985. Field and laboratory studies of the feeding behaviour of the piper *Hyporhamphus ihi* with reference to the role of the lateral line in feeding. *Proc. R. Soc. Lond.* **224**: 209–221.
Schluter, D. & Nagel, L.M. 1995. Parallel speciation by natural selection. *Am. Nat.* **146**: 292–301.
Shikano, T., Shimada, Y., Herczeg, G. & Merilä, J. 2010. History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. *Mol. Ecol.* **19**: 1147–1161.
Spiegelhalter, D.J., Best, N.G., Carlin, B.R. & van der Linde, A. 2002. Bayesian measures of model complexity and fit. *J. Roy. Stat. Soc. Ser. B-Stat. Method.* **64**: 583–616.
Spitze, K. 1993. Population-structure in *Daphnia obtusa*: quantitative genetic and allozymic variation. *Genetics* **135**: 367–374.
Taggart, J.B., Hynes, R.A., Prodöhl, P.A. & Ferguson, A. 1992. A simplified protocol for routine total DNA isolation from salmonid fishes. *J. Fish Biol.* **40**: 963–965.
Teyke, T. 1985. Collision with and avoidance of obstacles by blind cave fish *Anoptichthys jordani* (Characidae). *J. Comp. Physiol.* **157**: 843–849.
Van Netten, S. & Kroese, A.B.A. 1987. Laser interferometric measurements on the dynamic behavior of the cupula in the fish lateral line. *Hearing Res.* **29**: 55–62.
Wark, A.R. & Peichel, C.L. 2010. Lateral line diversity among ecologically divergent threespine stickleback populations. *J. Exp. Biol.* **213**: 108–117.
Webb, J.F. 1989. Gross morphology and evolution of the mechanoreceptive lateral-line system in teleost fishes. *Brain Behav. Evol.* **33**: 34–53.
Whitlock, M.C. 2008. Evolutionary inference from Q_{ST}. *Mol. Ecol.* **17**: 1885–1896.
Wright, S. 1931. Evolution in Mendelian populations. *Genetics* **16**: 97–159.
Ziuganov, V.V. & Zotin, A.A. 1995. Pelvic girdle polymorphism and reproductive barriers in the ninespine stickleback *Pungitius pungitius* from northwest Russia. *Behaviour* **132**: 1095–1105.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Canonical loadings for discriminant axes of between-class correspondence analyses.

Figure S1 Median number of neuromasts for each lateral line region, averaged by sex and habitat (marine vs. pond).

Figure S2 Median number of neuromasts for each lateral line region, averaged by experimental group (laboratory-reared vs. wild-caught) and habitat of origin (marine vs. pond).

Figure S3 Scatter plots of neuromast-specific P_{ST} and F_{ST} estimated for all pairwise population combinations.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be reorganized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

Received 25 January 2011; revised 19 March 2011; accepted 27 March 2011

BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

High levels of fluctuating asymmetry in isolated stickleback populations

Nina Trokovic, Gábor Herczeg, Nurul Izza Ab Ghani, Takahito Shikano and Juha Merilä*

Abstract

Background: Fluctuating asymmetry (FA), defined as small random deviations from the ideal bilateral symmetry, has been hypothesized to increase in response to both genetic and environmental stress experienced by a population. We compared levels of FA in 12 bilateral meristic traits (*viz*. lateral-line system neuromasts and lateral plates), and heterozygosity in 23 microsatellite loci, among four marine (high piscine predation risk) and four pond (zero piscine predation risk) populations of nine-spined sticklebacks (*Pungitius pungitius*).

Results: Pond sticklebacks had on average three times higher levels of FA than marine fish and this difference was highly significant. Heterozygosity in microsatellite markers was on average two times lower in pond ($H_E \approx 0.3$) than in marine ($H_E \approx 0.6$) populations, and levels of FA and heterozygosity were negatively correlated across populations. However, after controlling for habitat effect on heterozygosity, levels of FA and heterozygosity were uncorrelated.

Conclusions: The fact that levels of FA in traits likely to be important in the context of predator evasion were elevated in ponds compared to marine populations suggests that relaxed selection for homeostasis in ponds lacking predatory fish may be responsible for the observed habitat difference in levels of FA. This inference also aligns with the observation that the levels of genetic variability across the populations did not explain population differences in levels of FA after correcting for habitat effect. Hence, while differences in strength of selection, rather than in the degree of genetic stress could be argued to explain habitat differences in levels of FA, the hypothesis that increased FA in ponds is caused by genetic stress cannot be rejected.

Background

Three types of asymmetry in bilateral characters have been recognized: directional asymmetry, antisymmetry, and fluctuating asymmetry (FA) [1]. While both directional asymmetry (the same side is consistently larger) and antisymmetry (one of the sides is consistently larger) result from normal development, FA refers to subtle random deviations from perfect symmetry in bilateral traits resulting from developmental perturbations, and is often used as an indicator of stress and/or fitness e.g. [2-7]. The assumption underlying this practise is that FA reflects developmental instability (DI) – an organism's inability to adjust its development in an ideal symmetric pattern [8]. Several studies have shown that high FA levels are characteristics of individuals with low fitness e.g. [9-12]. The link between FA and various forms of stress has been repeatedly observed: habitat degradation [13], pollution [14], hybridisation [15], inbreeding [16], small population size [17], and marginal distribution [18] have all been associated with increased levels of FA. Therefore, FA has been proposed to be a useful bioindicator of individual quality and/or environmental stress e.g. [2-4]. However, despite these positive results, a number of studies have failed to find the expected relationships between FA and stress or fitness, fuelling a debate about the general applicability of FA as a bioindicator trait in conservation biology for reviews, see [6,19,20]. Numerous analytical and statistical issues, such as the proper control of measurement error in metric traits e.g. [21-23], and the difficulty of reliably estimating DI using single traits [6,7,23,24], might provide at least partial explanation for the conflicting results. These difficulties have also been proposed to account for the recent decrease in popularity of FA studies [6].

* Correspondence: juha.merila@helsinki.fi
Department of Biosciences, Ecological Genetics Research Unit, University of Helsinki, PO Box 65FI-00014, Helsinki, Finland

While both theory and a number of observations align with the idea that the degree of FA at the individual or population level is indicative of individual quality or degree of stress experienced, relaxed selection against developmental perturbations is also expected to increase FA in given population and/or trait. For instance, several studies have shown that the levels of FA in functionally important bilateral traits is typically less than that in functionally less important traits e.g. [25]. Similarly, it is possible that the degree of canalizing selection against developmental perturbations may differ among different populations. If so, this could provide one explanation for heterogeneity in FA-stress associations in different studies: in two populations experiencing the same incidence of stress induced developmental errors, the one experiencing relaxed selection against FA will express a higher degree of FA on average than a population that is under more stringent normalizing selection. However, to the best of our knowledge, this hypothesis has not been tested to date.

The mechanosensory lateral line system, present in all fishes and aquatic amphibians, has anatomical and functional properties which make it highly suitable and attractive for FA studies. Firstly, the lateral line system consists of numerous sensory receptors (neuromasts) located on the surface of the animal, either superficially (superficial or free neuromasts) or under the skin in fluid-filled canals (canal neuromasts) e.g. [26], which can be counted easily. Meristic traits, such as neuromasts, have been shown to be superior over metric traits in detecting correlations between FA and the environment [20], and can be counted with little error. Secondly, neuromasts are organised in anatomically distinct lines that are distributed bilaterally along the head and trunk, and the existence of multiple traits (i.e. individual lines) provides the possibility to determine the overall level of FA precisely, unlike most single-trait estimations [6]. Thirdly, the lateral line system is functionally very important, and likely to influence individual fitness. This system senses weak water movements and mediates crucial behaviours, including prey detection [27,28], predator avoidance [29], schooling [30], orientation to water currents (rheotaxis) [31], and localization of objects [32-34]. Hence, lateral line asymmetry is likely to reduce fitness, and as such, it is a potential target of natural selection. Further, this effect could be expected to differ among populations living in environments which differ in the demands on the lateral line system.

The goal of the present paper was to compare the degree of FA of marine (high piscine predation risk) and pond (zero piscine predation risk) nine-spined stickleback (*Pungitius pungitius*) populations differing both in the levels of genetic diversity [35] and in the level of expected selection (by piscine predation) for symmetry. Assuming that perfect symmetry in the lateral line system is favoured by natural selection, we hypothesised that either (i) the relaxed selection for symmetry in pond populations under negligible predation, and/or (ii) the reduced genetic variability (= genetic stress) in pond populations, will result in reduced developmental stability in ponds as compared to marine populations. In both cases, one would expect to see higher FA levels in pond than in marine populations. However, because genetic variability varies between populations within the same habitat [35], we also attempted to disentangle the two alternative explanations for increased FA in pond environments.

Results

The GLMM on heterozygosity revealed a significant habitat effect ($F_{1,6} = 10.17$, $P = 0.019$), but no population effect ($Z = 1.45$, $P = 0.15$). The average (± S.E.) heterozygosity in marine populations ($H_E = 0.58 \pm 0.06$) was approximately two times higher than in pond populations ($H_E = 0.30 \pm 0.06$; Figure 1). The GLM revealed a significant population effect ($F_{7,176} = 14.47$, $P < 0.001$) and subsequent post hoc tests revealed no heterogeneity among marine populations (all $P > 0.22$, Figure 1). The Mashinnoje pond population that has only recently become isolated from the White Sea [36]; White Sea Biological Station staff personal communication] did not differ from the marine populations in terms of heterozygosity (all $P > 0.13$, Figure 1). The remaining three ponds (Abbortjärn, Pyöreälampi and Rytilampi) had lower heterozygosity than the marine or Mashinnoje populations (all $P < 0.01$, Figure 1). Pyöreälampi had lower heterozygosity than any of the other populations (all $P < 0.004$, Figure 1). Therefore, while the marine populations had uniformly high heterozygosity, in the ponds heterozygosity varied from being similar to the marine levels (Mashinnoje) to almost zero (Pyöreälampi; Figure 1.).

The GLMM revealed a significant habitat effect ($F_{1,6} = 35.60$, $P < 0.001$) on the composite standardized relative FA-index, irrespective of sex ($F_{1,156} = 0.27$, $P = 0.60$), population ($Z = 1.05$, $P = 0.30$), and heterozygosity ($F_{1,5} = 2.19$, $P = 0.20$). None of the interactions were significant (habitat*sex: $F_{1,143} = 0.03$, $P = 0.86$; habitat*heterozygosity: $F_{1,4} = 0.10$, $P = 0.76$). The pond populations showed almost three times higher levels of asymmetry than the marine populations (Least Squares means ± S.E.; marine = 6.55 ± 1.3; pond = 17.45 ± 1.3; Figure 1). The multivariate GLM supported these results, revealing a strong habitat effect (Wilk's $\lambda_{12,141} = 0.59$, $P < 0.001$) on standardized relative asymmetry, irrespective of sex (Wilk's $\lambda_{12,140} = 0.91$, $P = 0.36$, habitat*sex: Wilk's $\lambda_{12,139} = 0.96$, $P = 0.91$). The population effect was also non-significant (Wilk's $\lambda_{72,773} = 0.54$, $P = 0.07$), and the univariate tests showed significant habitat effects for standardized relative asymmetry in all 12 traits ($F_{1,150} > 5.38$, $P < 0.02$). The trends

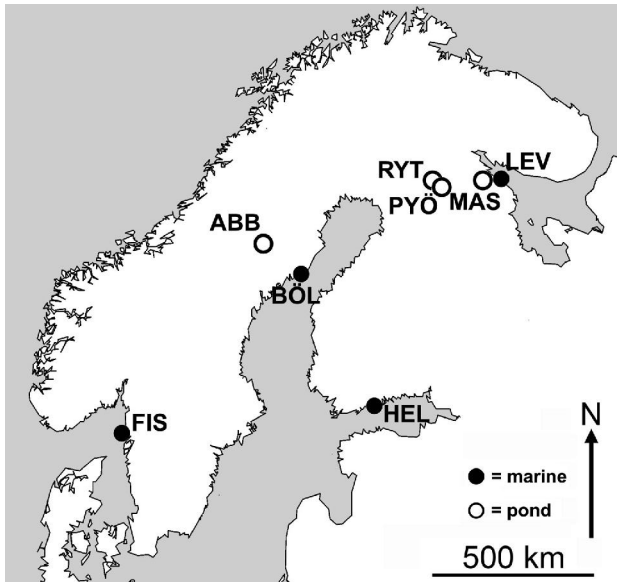


Figure 1 Average heterozygosity and mean fluctuating asymmetry in eight nine-spined stickleback (*Pungitius pungitius*) populations. Average (± S.E) heterozygosity was based on screening of variation in 23 microsatellite loci, and mean (± S.E.) fluctuating asymmetry on 12 bilateral traits as estimated by composite standardized relative asymmetry index (see Methods). For population abbreviations, see Figure 2.

were similar to those revealed by the composite relative asymmetry index: pond populations were generally more asymmetric than marine populations (data not shown).

To explore further the (lack of) heterozygosity effect on FA in the GLMM (see above), we performed simple correlation analyses using the population mean values of the two traits. Using raw values, there was strong negative correlation between mean FA and mean heterozygosity across the eight populations (r_s = - 0.833, P = 0.01). However, if the average effect of habitat type is controlled for by performing the correlation using heterozygosity values standardized to a common mean across the habitat types, this correlation disappears (r_s = - 0.071, P = 0.35). These results are compatible with the results of the GLMM above, and show that the association between FA and heterozygosity is mainly driven by the association between habitat type and heterozygosity.

Discussion

The most salient finding of this study was that nine-spined sticklebacks from ponds exhibit significantly and consistently higher levels of FA than their marine conspecifics. Furthermore, while the pond sticklebacks in general had only about half of the genetic variability of marine sticklebacks, the analyses did not support the idea that habitat differences in levels of FA are explainable by differences in heterozygosity once the habitat differences in heterozygosity are controlled for. Hence, the results support the conjecture that high levels of FA in pond populations stem from decreased selection for perfect symmetry, rather than from genetic stress.

Predation is a widely recognized mechanism of natural selection, and some studies have shown that predated individuals express higher levels of FA than surviving individuals e.g. [9,37-39]. Furthermore, decreased FA with age is also suggestive of poorer survival of more asymmetric individuals e.g.[40]. Predation can decrease the population level FA in the prey in at least three ways. First, predation can impose selection for individuals with low FA, resulting in a high degree of developmental canalization and thereby in low FA. However, this implies that there is an additive genetic basis for FA. Heritability of FA is a controversial issue: initial meta-analyses yielded a relatively high average heritability estimate

[41], but the subsequent studies have since suggested that the heritability of FA is very low – if not negligible [42,43]. Second, assuming that FA has no, or a weak, genetic basis, and is simply a reflection of the growth environment experienced, predation might simply remove asymmetric individuals from the population. Third, as negligible predation selects for larger body size and higher growth rate [44,45] predation has the potential to affect FA indirectly through altering the growth intensity, where higher growth rate is coupled with higher DI [46]. Regardless of the mechanism, relaxation of predation pressure can be expected to increase the average degree of FA in the population. While we are not aware of any studies that have compared FA levels among populations that differ in selection for perfect symmetry, there are studies which show that less functionally important traits express higher FA than important traits at the individual level e.g. [25]. That said, it is also known that strong directional selection can increase DI in selected traits [47]. However, although the mean number and organisation of lateral-line neuromast differ among marine and pond populations in this species [48], the patterns of differentiation among populations are heterogeneous and directional selection on lateral-line traits is indicated to occur mainly in the marine environment [48]. Likewise, the lateral plate number – one of the traits analysed in study – is shown to be reduced in pond as compared marine populations presumably as response relaxed piscine predation in pond environments [49]. Hence, it seems unlikely that the increased DI in pond populations' lateral-line traits and lateral plate numbers would results from directional selection.

Perhaps the most marked difference between pond and marine nine-spined stickleback populations is the predation risk; marine sticklebacks are sympatric to a large number of predatory fish species, while ponds lack predatory fish and the nine-spined stickleback is often the only fish species present in ponds [49,50]. Previous studies have demonstrated marked behavioral and morphological differences in nine-spined sticklebacks in relation to predation risk [44,49,50], including a recent study demonstrating habitat and population specific differences in the lateral line system [48]. The mechanosensory lateral line system of fish and aquatic amphibians responds to weak water movements and is involved in avoidance of predators [29,49,51] and in schooling [30], which is an important antipredator behaviour [52]. Hence the negligible predation in ponds might have resulted in relaxed selection for perfect symmetry in the lateral line system and consequently, in the high levels of FA observed in this habitat. While this is, to the best of our knowledge, the first study to suggest this effect, we admit that the exact functions of the different lateral-line traits in this species are as yet unknown [26,48]. Hence, further functional studies about how the information from the lateral-line is used in different contexts are needed. However, the fact that levels of FA in lateral plate numbers – a trait associated with variation in piscine predation [49] – showed exactly the same patterns of FA as lateral-line traits supports the importance of predation in dictating the observed patterns.

Inbreeding (mating among relatives) can increase homozygosity and result in inbreeding depression, which can manifest itself in reduced survival and fertility e.g. [52,53]. Increased FA levels have been linked to reduced heterozygosity both in the field e.g. [16,54,55], and in controlled laboratory experiments with induced inbreeding e.g. [56]. It has been shown that pond nine-spined stickleback populations have lower genetic variability than marine populations [35], and one explanation for the higher level of FA in pond sticklebacks could be that reduced genetic variability in pond populations has resulted in increased DI, and consequently increased FA. Based on the populations used in this study, the heterozygosity of pond populations was on average half that of marine populations, with heterozygosity being highly variable among pond populations, but similar among marine populations. However, formal tests – accounting for the on average lower heterozygosity in pond populations – failed to find association between heterozygosity and FA across the populations. This finding is not completely surprising, as some other studies also found that heterozygosity had a weak, or no effect on FA [57-60]. However, given the fact heterozygosity and habitat type are tightly associated in our study, their independent effects on FA cannot be fully disentangled.

Obviously, there are other factors that potentially affect FA that we could not directly address here. For instance, there might be environmental stressors (e.g. water quality, temperature, oxygen levels, pH, etc.) that may differ among marine and pond populations, and cause higher levels of FA in ponds. At the moment, too little is known about the variation in relevant environmental parameters and their potential impact on FA in pond vs. marine habitats to form informed arguments about their significance, but it is worth noting that there is no *a priori* reason to suggest that pond fish would experience more stressful environmental than the marine fish. In fact, pond fish live longer, grow faster and attain larger sizes than marine fish both in laboratory and the wild [44,45]. Nevertheless, more environmental data coupled with experiments conducted under common garden settings would be needed to study possible environmental determinants of high FA in pond fish. However, irrespectively of the causes, the fact remains that the levels of FA in pond populations are markedly elevated as compared to marine populations.

Conclusions

In conclusion, the results demonstrate that there is a three-fold difference in levels of FA between pond and marine nine-spined stickleback populations (pond > marine). While there is also a two-fold difference in heterozygosity (pond < marine), the loss of genetic variation did not explain the divergence in levels of FA once the habitat differences in heterozygosity were controlled for. We hypothesize that the negligible predation in pond populations (contrasted to the high predation in marine environments) is responsible for the increased FA in ponds.

Methods

Study populations and data collection

The nine-spined stickleback is a small-bodied teleost fish with a circumpolar distribution, which occurs in various habitats that differ with respect to both biotic and abiotic stress [61]. Adult nine-spined sticklebacks were collected using minnow traps and seine nets during the breeding seasons (May- June) between 2007 and 2009. The fish were collected from four ponds and four marine populations from geographically distinct locations in Fennoscandia (Figure 2). Coastal marine environments represent ecologically complex habitats with diverse fish communities, and a large number of potential predator fish species that can predate on every age and size group of nine-spined sticklebacks, while ponds, which are extremely small (surface area < 5 ha) and completely isolated, lack predatory fish [44]. The only sympatric fish species in our study ponds was the three-spined stickleback (*Gasterosteus aculeatus*) in Mashinnoje, and recently introduced small-bodied whitefish (*Coregonus lavaretus*) in Pyöreälampi, both of which are potential competitors, but not predators, of nine-spined sticklebacks. We note that apart from predation by fish, sticklebacks are also predated by aquatic insects, birds and conspecifics. However, according to our observations, bird predators in our study ponds are extremely scarce if not absent, whereas they are numerous at the marine sites [Gábor Herczeg & Juha Merilä personal observations]. Predation by aquatic insects and adult conspecifics might be relevant in all populations to a certain degree; we have no quantitative data on these effects. However, several lines of independent evidence suggest that the predation regime in pond and marine populations differ drastically. Namely, pond sticklebacks have reduced or absent defensive body armour, live almost two times longer on average, and behave bolder when compared to marine fish [44,49,50]. Hence, the difference in predation risk by piscine predators appears to be defining feature differentiating marine and pond populations in focus of this study.

Collected fish were over-anesthetized with MS 222 (tricaine methanesulfonate) at the site of capture, and stored in 96 % ethanol. Samples were later fixed in 4 % formalin. A standard bone-staining procedure was used for the visualization of neuromasts. In short, the fish samples were briefly dehydrated in 70 % ethanol, and placed in 1 g/l alizarin red; 0.5 % KOH for 3 days. Fish were destained in 1 % KOH for 4 days and transferred to alcohol. Posterior lateral plates and neuromasts from 11 lateral lines (Figure 3) were counted under a dissecting microscope (Wild M5A; Wild, Heerbrugg, Switzerland). The use of meristic characters instead of metric ones is beneficial, because the former can be recorded (in theory) with perfect accuracy, while the latter can not. We used 20 individuals from every population. We aimed to measure 10 males and 10 females per population, but in two marine populations (Helsinki, Levin Navolok Bay; see Figure 2) we only obtained females, so we used 20 females for these populations. Twenty individuals were counted twice for every trait. The repeatability (R) of the left side – right side values were high (mean R = 0.97, median R = 1, minimum-maximum: 0.75 – 1, $P < 0.001$ in all tests).

Since this was a non-experimental study involving only collection of animals, no ethical permissions were required. Nine-spined stickleback is not protected in any of the sampled countries, hence, sampling permits from local conservation authorities were not needed in

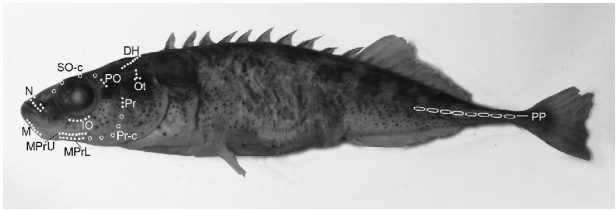


Figure 2 Map of Fennoscandia showing the localities of the nine-spined stickleback (*Pungitius pungitius*) populations used in our study. FIS = Fiskebäckskil, Atlantic Ocean, Sweden; BÖL = Bölesviken, Baltic Sea, Sweden; HEL = Helsinki, Baltic Sea, Finland; LEV = Levin Navolok Bay, White Sea, Russia; ABB = Abbortjärn, Sweden; RYT = Rytilampi, Finland; PYÖ = Pyöreälampi, Finland; MAS = Mashinnoje, Russia.

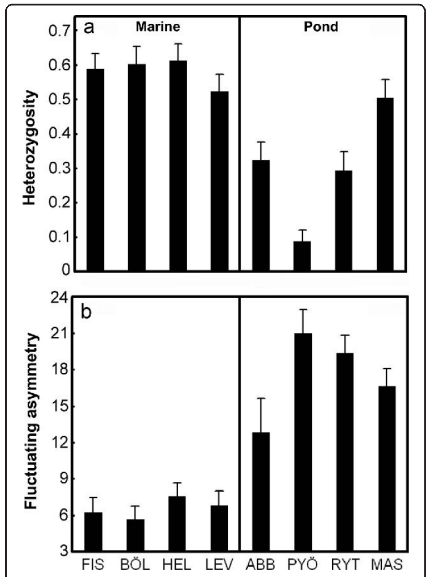


Figure 3 Schematic presentation of the location of the lateral line and lateral plate traits. Canal neuromast pores are shown as open circles, superficial neuromasts as dots. Shown are also posterior plates (PP), and lateral line structures: dorsal head (DH), infraorbital (IO), mandibular (M), mandibular preopercular lower (MPrL), mandibular preopercular upper (MPrU), nasal (N), otic (Ot), postorbital (PO), preopercular (Pr), preopercular canal (Pr-c), supraorbital canal (SO-c).

general. However, one of the sampled areas (Oulanka region in Finland) included a protected area, and for sampling in the water bodies in that particular area, we requested and received sampling permit from the responsible conservation authority, Metsähallitus (2007, permit given for GH). To import the samples to Finland, we requested and received permits from the Finnish Food Safety Authority (EVIRA, # 2736/425/2007, 3342/425/2007, 577/0527/2009).

Genetic analyses

DNA was extracted from fin clips using a phenol-chloroform [62] or a silica-fine based purification method [63], following proteinase K digestion. The following 23 microsatellite loci were genotyped: Gac1125PBBE, Gac4174PBBE, Gac7033PBBE, GAest3, GAest7, GAest14, GAest35, GAest50, GAest66, GAest82, Stn49, Stn71, Stn89, Stn96, Stn100, Stn127, Stn130, Stn163, Stn173, Stn196, Stn198, Stn223 and Stn253 [64-68]. The forward primers were labelled with fluorescent dyes (FAM, HEX or TET) for visualization of the PCR products, and the 5'-end of the reverse primers was modified with an additional GTTT-tail to enhance the 3'-adenylation [69]. The loci were arranged in multiplex PCR panels with non-overlapping size ranges, and all amplifications were carried out using the Qiagen Multiplex PCR Kit (Qiagen) containing 1× Qiagen Multiplex PCR Master Mix, 0.5× Q-Solution, 2 ρmol of each primer, 10–20 ng of template DNA and MQ water for a final reaction volume of 10 μl. PCRs were performed using the following cycle: an initial denaturation step at 95 °C for 15 min, followed by 30 s at 94 °C, 90 s at 53 °C and 60 s at 72 °C for 30 cycles, with a final extension at 60 °C for 5 min. PCR products were separated using a MegaBACE 1000 automated sequencer (Amersham Biosciences) and their sizes were determined using ET-ROX 400 or 550 size standard (Amersham Biosciences). Alleles were scored using Fragment Profiler 1.2 (Amersham Biosciences), with visual inspection and manual correction. Some (11 loci for seven populations) of the microsatellite genotypic data used in here are a subset of data from Shikano *et al.* [35]. Genetic diversities (H_E) were estimated using FSTAT 2.9.3 [70]. To verify that the used genetic markers behave as neutral, we also conducted an outlier test using the program LOSITAN [71]. The results suggest that none of the used microsatellite loci have been subject to recent directional selection. Although three loci (GAest14, GAest50 and GAest66) were indicated to be under balancing selection, we retained them in the analyses because of the methodological problems associated with the identification of balancing selection in outlier tests [72]. We also note that our estimates of heterozygosity in 23 microsatellite loci are likely to reflect true genome-wide heterozygosity [c.f. [73] in these populations: correlation between heterozygosity in the 12 of the microsatellite loci also used here and 15 318 SNP loci across eight nine-spined stickleback populations has been found to be very high (r = 0.97; Johnston S., et al, submitted]). Likewise, mean heterozygosity in the 23 markers used in this study is strongly correlated with mean heterozygosity in abovementioned SNPs across five populations common with this and the SNP study (r = 0.92). Furthermore, a subset (n = 5) of populations used in this study have also been genotyped for 112 microsatellite loci (T. Shikano, unpublished data) and the correlation between estimates based on 112 *vs.* 23 markers is high (r = 0.96). Hence, the population specific heterozygosity estimates are likely to be good estimators of genome-wide heterozygosity.

Statistical analyses

To compare the levels of heterozygosity between the populations, we used locus- and population-specific heterozygosity estimates. First, we used a General Linear Mixed Model (GLMM) to test for habitat effects. Here,

heterozygosity estimated for the different loci was the dependent variable, habitat type (marine *vs.* pond) the fixed factor, and population nested within habitat a random factor. Second, to have population-based pairwise comparisons, we ran a General Linear Model (GLM) with heterozygosity as the dependent variable and population as a fixed factor, followed by pairwise Fisher LSD post hoc tests.

To ensure that the analysed asymmetry is FA, one has to exclude the possibility that directional asymmetry and antisymmetry [see e.g. 1] are responsible for the patterns. To test for the presence of directional asymmetry, we compared the left side–right side values by trait and population to the hypothetical zero value using one-sample t-tests. Out of the 96 tests, only four cases were significant (data not shown). Considering that the four significant cases represented (i) different traits in different populations, (ii) only 4.2 % of all tests, and (iii) there were 12 non-independent tests for every population, we can be confident that the measured asymmetry was not directional asymmetry. The presence of antisymmetry was excluded after visual inspection of the distributions of the left – right side values for every trait and every population separately.

We performed two analyses to look at FA with multiple traits. For the first analysis, we calculated a composite FA-index for each individual based on all traits. This index was modified after Leung et al.'s [7] recommended Composite Fluctuating Asymmetry index 2 (CFA 2). We initially calculated relative asymmetry to take the mean number of counts for a given trait into account [74,75]:

$$RA_{i,j} = \frac{\left|L_{i,j} - R_{i,j}\right|}{\frac{L_{i,j}+R_{i,j}}{2}} \quad (1)$$

where the relative asymmetry (*RA*) for individual *i*'s trait *j* is given based on the left (*L*) and right hand (*R*) counts. This step is important because there is a big developmental difference between hypothetical cases with one vs. two, or 101 vs. 102, counts in the two sides. Using this *RA* instead of the absolute difference between the sides, we then followed Leung et al.'s [7] CFA 2 procedure by first dividing individual *RA* values with the mean *RA* found in the given trait across all individuals to control for possible differences in the relationship between FA and DI in different traits:

$$SRA_{i,j} = RA_{i,j}/\overline{RA}_j \quad (2)$$

and then summarized these standardized relative asymmetry values (*SRA*) across all traits for every individual:

$$CSRA_i = \sum SRA_i \quad (3)$$

Composite standardized relative asymmetry (*CSRA*) describes the individual level asymmetry considering every trait with equal weight. *CSRA* was then analysed with a GLMM with habitat (marine *vs.* pond) and sex as fixed factors, heterozygosity as a covariate, and population nested within habitat as a random factor.

For the second analysis, we ran a multivariate GLM with the trait-based *SRA* (2) values as dependent variables, and with habitat, sex and population nested within habitat as fixed factors. Upon significant multivariate effects, we evaluated the subsequent univariate tests. Note that no random factor can be fitted in multivariate models, and hence, we had to enter population nested within habitat as a fixed factor. For this reason we could not test for the effects of (population level) heterozygosity and habitat in the same model, and thus had to drop heterozygosity from this second approach. However, as simulations have shown that the multivariate GLM (MANOVA) approach is inferior compared to the use of standardized, summed FA values across traits similar to our *CSRA* [7], we only used the multivariate GLM as a means to see whether the habitat-dependent patterns revealed by the GLMM on *CSRA* (see Results, heterozygosity had no effect) can be found on a trait-by-trait basis too.

In all models, we included the habitat × sex interaction, and in the GLMM, the habitat × heterozygosity interaction was also included. To avoid misinterpretation of the data, the non-significant terms from the models were removed e.g. [76] using backward stepwise models selection based on the $P < 0.05$ criterion see e.g. [77]. This method for model selection is conservative in comparison with those based on Akaike's or Bayesian information criteria (AIC or BIC), and differs very little from the others in its predictive ability [78]. We mentioned earlier that two of the eight populations lacked males, and so the sex effects should be interpreted accordingly. All analyses were done using SPSS 15.0 for Windows (SPSS Inc. Chicago, Illinois).

Competing interests
The authors declare no competing interests.

Authors' contributions
NT conducted the lateral line counting and conceived and designed the study together with JM and GH. GH and JM collected the samples. TS and NIAG carried out the genetic analyses. GH, NT and JM conducted the statistical analyses. NT and JM finalized the manuscript draft with help from other authors. All authors read and approved the final manuscript.

Acknowledgements
We thank Victor Berger, Göran Englund, Jaquelin DeFaveri, Abigél Gonda, Lotta Kvarnström, Tuomas Leinonen, and Jouko Pokela for help in obtaining the samples, Marika Karjalainen and Kirsi Kähkönen for their assistance in the laboratory and Amber Teacher for commenting earlier draft of this manuscript. Our study was supported by the Academy of Finland (to J.M., G. H, and T.S.), the Japan Society for the Promotion of Science (to T.S.), Universiti Putra Malaysia (to N.I.A.G.), and Ella and Georg Ehrnrooth foundation (to N.T.). The research leading to these results received also funding from the European Community's Seventh Framework Programme (FP/2007-2013) under grant agreement n° 217246 made with BONUS, the joint Baltic Sea research and development programme (to J.M.).

Received: 11 April 2012 Accepted: 12 July 2012
Published: 12 July 2012

References
1. Van Valen L: A study of fluctuating asymmetry. *Evolution* 1962, 16:125–142.
2. Leary RF, Allendorf FW: Fluctuating asymmetry as an indicator of stress: implications for conservation biology. *Trends Ecol Evol* 1989, 4:214–217.
3. Parsons PA: Fluctuating asymmetry: a biological monitor of environmental and genomic stress. *Heredity* 1992, 68:361–364.
4. Clarke GM: Relationships between developmental stability and fitness: application for conservation biology. *Cons Biol* 1995, 9:18–24.
5. Leung B, Forbes MR: Fluctuating asymmetry in relation to stress and fitness: effects of trait type as revealed by meta-analysis. *Ecoscience* 1996, 3:400–413.
6. Van Dongen S: Fluctuating asymmetry and developmental instability in evolutionary biology: past, present and future. *J Evol Biol* 2006, 19:1727–1743.
7. Leung B, Forbes MR, Houle D: Fluctuating asymmetry as a bioindicator of stress: comparing efficacy of analyses involving multiple traits. *Am Nat* 2000, 155:101–115.
8. Palmer AR, Strobeck C: Fluctuating asymmetry: measurement, analysis, patterns. *Ann Rev Ecol Syst* 1986, 17:391–421.
9. Møller AP: Sexual selection, viability selection and developmental stability in the domestic fly *Musca domestica*. *Evolution* 1996, 50:746–752.
10. Martín J, López P: Chemoreception, symmetry and mate choice in lizards. *Proc Roy Soc Lond B* 2000, 267:1265–1269.
11. Martín J, López P: Hindlimb asymmetry reduces escape performance in the lizard *Psammodromus algirus*. *Physiol Biochem Zool* 2001, 74:619–624.
12. Polak M, Taylor PW: A primary role of developmental instability in sexual selection. *Proc Roy Soc Lond B* 2007, 274:3133–3140.
13. Sarre S: Habitat fragmentation promotes fluctuating asymmetry but not morphological divergence in two geckos. *Res Pop Ecol* 1996, 38:57–64.
14. Bengtsson BE, Bengtsson A, Himberg M: Fish deformities and pollution in some Swedish water. *Am Biol* 1985, 14:32–35.
15. Graham JH, Felley JD: Genomic coadaptation and developmental stability with introgressed populations of *Enneacanthus gloriosus* and *E. obesus*. *Evolution* 1985, 39:72–87.
16. Roldan RS, Cassinello T, Abaigar T, Gomeindo M: Inbreeding, fluctuating asymmetry and ejaculate quality in an endangered ungulate. *Proc Roy Soc Lond B* 1998, 265:243–248.
17. Hutchinson DW, Cheverud JM: Fluctuating asymmetry in tamarin (*Saguinus*) cranial morphology: intra- and interspecific comparisons between taxa with varying levels of genetic heterozygosity. *J Hered* 1995, 86:280–288.
18. Siikamäki P, Lammi A: Fluctuating asymmetry in central and marginal populations of *Lychnis viscaria* in relation to genetic and environmental factors. *Evolution* 1998, 52:1285–1292.
19. Lens L, van Dongen S, Kark S, Matthysen E: Fluctuating asymmetry as an indicator of stress: can we bridge the gap between studies? *Biol. Rev* 2002, 77:27–38.
20. Lens L, Van Dongen S, Kark S, Talloen W, Hens L, Matthysen E: The use of bilateral asymmetry in ecology and conservation: concept, developments and prospects. *Recent Res Dev Ecol* 2001, 1:21–41.
21. Merilä J, Björklund M: Fluctuating asymmetry and measurement error. *Syst Biol* 1995, 44:97–101.
22. Palmer AR: Detecting publication bias in meta-analyses: a case study in of fluctuating asymmetry and sexual selection. *Am Nat* 1999, 154:220–233.
23. Palmer M, Linde M, Morales-Nin B: Disentangling fluctuating asymmetry from otolith shape. *Mar Ecol Prog Ser* 2010, 399:261–272.
24. Van Dongen S, Lens L, Pape E, *et al*: Evolutionary history shapes the association between developmental instability and population-level genetic variation in three-spined sticklebacks. *J Evol Biol* 2009, 22:1695–1707.
25. Karvonen E, Merilä J, Rintamaki PT, Van Dongen S: Geography of Xuctuating asymmetry in the greenfinch, *Carduelis chloris*. *Oikos* 2003, 100:507–516.
26. Honkanen T: Comparative study of the lateral-line system of the 3-spined stickleback (*Gasterosteus aculeatus*) and the 9-spined stickleback (*Pungitius pungitius*). *Acta Zool* 1993, 74:331–336.
27. Montgomery JC, Macdonald JA: Sensory tuning of lateral line receptors in Antarctic fish to the movements of planktonic prey. *Science* 1987, 235:195–196.
28. Montgomery JC, Milton RC: Use of the lateral line for feeding in the torrentfish (*Cheimarrichthys fosteri*). *New Zealand J Zool* 1993, 20:121–125.
29. Fuiman LA: Development of predator evasion in Atlantic herring. *Clupea harengus L. Anim Behav* 1993, 45:1101–1116.
30. Partridge BL, Pitcher TJ: The sensory basis of fish schools, relative roles of lateral line and vision. *J Comp Physiol* 1980, 135:315–325.
31. Montgomery JC, Baker CF, Carton AG: The lateral line can mediate rheotaxis in fish. *Nature* 1997, 389:960–963.
32. Campenhausen C, Riess I, Weissert R: Detection of stationary objects by the blind cavefish *Anoptichthys jordani* (Characidae). *J Comp Physiol* 1981, 143:369–374.
33. Teyke T: Collision with and avoidance of obstacles by blind cavefish *Anoptichthys jordani* (Characidae). *J Comp Physiol* 1985, 157:843–849.
34. Hassan ES: On the discrimination of spatial intervals by the blind cavefish (*Anoptichthys jordani*). *J Comp Physiol* 1986, 159:701–710.
35. Shikano T, Shimada Y, Herczeg G, Merilä J: History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. *Mol Ecol* 2010, 19:1147–1161.
36. Ziuganov VV, Zotin AA: Pelvic girdle polymorphism and reproductive barriers in the ninespine stickleback *Pungitius pungitius* (L.) from northwest Russia. *Behaviour* 1995, 132:1095–1105.
37. Swaddle JP: Developmental stability and predation success in an insect predator-prey system. *Behav Ecol* 1997, 8:433–436.
38. Bergstrom CA, Reimchen TE: Asymmetry in structural defenses: insights into selective predation in the wild. *Evolution* 2003, 57:2128–2138.
39. Galeotti P, Sacchi R, Vicaño V: Fluctuating asymmetry in body traits increases predation risks: tawny owl selection against asymmetric woodmice. *Evol Ecol* 2005, 19:405–418.
40. Moran P, Izquierdo JI, Pendas AM, GarciaVazquez E: Fluctuating asymmetry and isozyme variation in Atlantic salmon: relation to age of wild and hatchery fish. *Trans Am Fish Soc* 1997, 126:194–199.
41. Møller AP, Thornhill R: A meta-analysis of the heritability of developmental stability. *J Evol Biol* 1997, 10:1–16.
42. Leamy LJ: Is developmental stability heritable? *J Evol Biol* 1997, 10:21–29.
43. Fuller RC, Houle D: Inheritance of developmental instability. In *Developmental Instability: Causes and Consequences*. Edited by Polak M. Oxford: Oxford University Press; 2003:157–186.
44. Herczeg G, Gonda A, Merilä J: Evolution of gigantism in nine-spined sticklebacks. *Evolution* 2009, 63:3190–3200.
45. Herczeg G, Gonda A, Kuparinen A, Merilä J: Contrasting growth strategies of pond vs. marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? *Evol Ecol* 2012, 26:109–122.
46. Mangel M, Munch SB: A life-history perspective of short- and long-term consequences of compensatory growth. *Am Nat* 2005, 166:E155–E176.
47. Leamy LJ, Klingenberg CP: The genetics and Evolution of fluctuating asymmetry. *Annu Rev Ecol Syst* 2005, 36:1–21.
48. Trokovic N, Herczeg G, McCairns RJS, Ab Ghani NI, Merilä J: Intraspecific divergence in the lateral line system in the nine-spined stickleback (*Pungitius pungitius*). *J Evol Biol*. In press.
49. Herczeg G, Turtiainen M, Merilä J: Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. *Biol J Linn Soc* 2010, 101:403–416.
50. Herczeg G, Gonda A, Merilä J: Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *J Evol Biol* 2009, 22:544–552.
51. Blaxter JHS, Fuiman LA: The role of the sensory systems of herring larvae in evading predatory fishes. *J Mar Biol* 1990, 70:413–427.
52. Larsson M: Possible functions of the octavolateralis system in fish schooling. *Fish Fish* 2009, 10:344–353.
53. Charlesworth D, Charlesworth B: Inbreeding depression and its evolutionary consequences. *Annu Rev Ecol Syst* 1987, 18:237–268.
54. Charlesworth D, Willis JH: The genetics of inbreeding depression. *Nat Rev* 2009, 10:783–796.

55. Leary RF, Allendorf FW, Knudson RL: **Developmental stability and enzyme heterozygosity in rainbow trout.** *Nature* 1983, **301**:71–72.
56. Quattro JM, Vrijenhoek RC: **Fitness differences among remnant populations of the endangered Sonoran Topminnow.** *Science* 1989, **245**:976–978.
57. Mazzi D, Lardiadèr CR, Bakker TCM: **Inbreeding and developmental stability in three-spined sticklebacks (*Gasterosteus aculeatus* L.).** *Heredity* 2002, **89**:293–299.
58. Clarke GM, Oldroyd BP, Hunt P: **The genetic basis of developmental stability in *Apis mellifera*: Heterozygosity versus genic Balance.** *Evolution* 1992, **46**:753–762.
59. Whitlock M: **Lack of correlation between heterozygosity and fitness in forked fungus beetles.** *Heredity* 1993, **70**:574–581.
60. Britten HB: **Meta-analyses of the association between multilocus heterozygosity and fitness.** *Evolution* 1996, **50**:2158–2164.
61. Bănărescu PM, Paepke HJ: *The Freshwater Fishes of Europe. Vol. 5/II.* Wiebelsheim: AULA Verlag; 2001.
62. Taggart JB, Hynes RA, Prodöhl PA, Ferguson A: **A simplified protocol for routine total DNA isolation from salmonid fishes.** *J Fish Biol* 1992, **40**:963–965.
63. Elphinstone MS, Hinten GN, Anderson MJ, Nock CJ: **An inexpensive and high-throughput procedure to extract and purify total genomic DNA for population studies.** *Mol Ecol Notes* 2003, **3**:317–320.
64. Largiadèr CR, Fries V, Kobler B, Bakker TC: **Isolation and characterization of microsatellite loci from the three-spined stickleback (*Gasterosteus aculeatus* L.).** *Mol Ecol* 1999, **8**:342–344.
65. Peichel CL, Nereng K, Ohgi KA, *et al*: **The genetic architecture of divergence between threespine stickleback species.** *Nature* 2001, **414**:901–905.
66. Colosimo PF, Peichel CL, Nereng K, *et al*: **The genetic architecture of parallel armor plate reduction in threespine sticklebacks.** *PLoS Biol* 2004, **2**:635–641.
67. Mäkinen HS, Cano JM, Merilä J: **Identifying footprints of directional and balancing selection in marine and freshwater three-spined stickleback (*Gasterosteus aculeatus*) populations.** *Mol Ecol* 2008, **17**:3565–3582.
68. Shikano T, Ramadevi J, Shimada Y, Merilä J: **Utility of sequenced genomes for microsatellite marker development in non-model organisms: a case study of functionally important genes in nine-spined sticklebacks (*Pungitius pungitius*).** *BMC Genom* 2010, **11**:334.
69. Brownstein MJ, Carpten JD, Smith JR: **Modulation of non-templated nucleotide addition by *Taq* DNA polymerase: primer modifications that facilitate genotyping.** *Biotechniques* 1996, **20**:1004–1010.
70. Goudet J: **FSTAT: a computer program to estimate and test gene diversities and fixation indices (version 2.9.3).** 2001, Available at http://www2.unil.ch/popgen/softwares/fstat.htm.
71. Antao T, Lopes A, Lopes RJ, Beja-Pereira A, Luikart G: **LOSITAN: A workbench to detect molecular adaptation based on a F_{st}-outlier method.** *BMC Bioinform* 2008, **9**:323.
72. Excoffier L, Hofer T, Foll M: **Detecting loci under selection in a hierarchically structured population.** *Heredity* 2009, **103**:285–298.
73. Väli U, Einarsson A, Waits L, Ellegren H: **To what extent do microsatellite markers reflect genome-wide genetic diversity in natural populations?** *Molecular Ecology* 2008, **17**:3807–3817.
74. Werner YL, Rothenstein D, Sivan N: **Directional asymmetry in reptiles (Sauria: Gekkonidae: *Ptyodactylus*) and its possible evolutionary role, with implications for biometrical methodology.** *J Zool Lond* 1991, **225**:647–658.
75. Herczeg G, Szabó K, Korsós Z: **Asymmetry and population characteristics in dice snakes (*Natrix tessellata*): an interpopulation comparison.** *Amphibia-Reptilia* 2005, **26**:422–426.
76. Engqvist L: **The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies.** *Anim Behav* 2005, **70**:967–971.
77. Török J, Hegyi G, Tóth L, Könczey R: **Unpredictable food supply modifies costs of reproduction and hampers individual optimization.** *Oecol* 2004, **141**:432–443.
78. Murtaugh PA: **Performance of several variable-selection methods applied to real ecological data.** *Ecol Lett* 2009, **12**:1061–1068.

doi:10.1186/1471-2148-12-115
Cite this article as: Trokovic *et al.*: High levels of fluctuating asymmetry in isolated stickleback populations. *BMC Evolutionary Biology* 2012 **12**:115.

Journal of Animal Ecology

Journal of Animal Ecology 2012, **81**, 859–867 doi: 10.1111/j.1365-2656.2012.01971.x

Ontogenetic and evolutionary effects of predation and competition on nine-spined stickleback (*Pungitius pungitius*) body size

Kaisa Välimäki* and Gábor Herczeg

Ecological Genetics Research Unit, Department of Biosciences, University Helsinki, PO Box 65, Helsinki FI-00014, Finland

Summary

1. Individual- and population-level variation in body size and growth often correlates with many fitness traits. Predation and food availability are expected to affect body size and growth as important agents of both natural selection and phenotypic plasticity. How differences in predation and food availability affect body size/growth during ontogeny in populations adapted to different predation and competition regimes is rarely studied.

2. Nine-spined stickleback (*Pungitius pungitius*) populations originating from habitats with varying levels of predation and competition are known to be locally adapted to their respective habitats in terms of body size and growth. Here, we studied how different levels of perceived predation risk and competition during ontogeny affect the reaction norms of body size and growth in (i) marine and pond populations adapted to different levels of predation and competition and (ii) different sexes. We reared nine-spined stickleback in a factorial experiment under two levels of perceived predation risk (present/absent) and competition (high/low food supply).

3. We found divergence in the reaction norms at two levels: (i) predation-adapted marine stickleback had stronger reactions to predatory cues than intraspecific competition-adapted pond stickleback, the latter being more sensitive to available food than the marine fish and (ii) females reacting more strongly to the treatments than males.

4. The repeated, habitat-dependent nature of the differences suggests that natural selection is the agent behind the observed patterns. Our results suggest that genetic adaptation to certain environmental factors also involves an increase in the range of expressible phenotypic plasticity. We found support for this phenomenon at two levels: (i) across populations driven by habitat type and (ii) within populations driven by sex.

Key-words: growth, local adaptation, phenotypic plasticity, sex-specific differences

Introduction

Evolutionary ecologists seek to understand the ultimate and proximate causes of phenotypic variation between individuals, populations and species. Variation in local environmental characteristics can cause phenotypic divergence between populations of the same species in two main ways. First, differences in abiotic and biotic environmental conditions can result in non-genetic environmentally induced differences (i.e. phenotypic plasticity; West-Eberhard 2003). Secondly, the same environmental conditions can also result in selection and lead to local adaptations during the course of evolution (i.e. adaptive [genetic] divergence; Kawecki & Ebert 2004). The relationship between phenotypic plasticity and local adaptation has been (e.g. Via *et al.* 1995; DeWitt & Scheiner 2004; de Jong 2005; Pigliucci, Murren & Schlichting 2006; Crispo 2007, 2008), and is still (Fusco & Minelli 2010; Pfennig *et al.* 2010), vigorously discussed. However, in many cases, both plastic and genetic variation can have a role in adaptive phenotypic change (Conover & Schultz 1995; Conover, Duffy & Hice 2009). Theoretical models have suggested that environmental variation (spatial and temporal) is an important factor in maintaining plasticity in populations (see e.g. Via & Lande 1985; Gomulkiewicz & Kirkpatrick 1992; Moran 1992; Ernande & Dieckmann 2004), especially when variation is predictable. On the other hand, plasticity is limited by the costs of expressing a plastic trait and maintaining the capacity of plasticity (DeWitt, Sih & Wilson 1998; Van Buskirk & Steiner 2009; Auld, Agrawal & Relyea 2010). Therefore, if plasticity is an evolving trait and there are costs involved in its maintenance and expression, directional selection may favour a high degree of plasticity towards environmental factors that are both relevant and show variation in the given environment. However, in constant environments, plasticity might be lost as a result of genetic drift (Crispo 2007), mutational degradation (Masel, King & Maughan 2007) or through genetic assimilation, which is a form of canalization where a plastic trait becomes fixed and environmental cues are no longer needed for its expression (Crispo 2007).

*Correspondence author. E-mail: kaisa.valimaki@helsinki.fi

Body size and growth are among the most important traits of interest in ecology and evolutionary biology. A large body of data shows that body size is strongly correlated with many physiological and fitness traits (Peters 1983; Roff 1992; Stearns 1992). On evolutionary time-scale, fecundity selection, male–male competition and female mate-choice are factors promoting larger body size (Wooton 1979; Clutton-Brock, Guinnes & Albon 1982; Shine 1988, 1989; Andersson 1994). On the other hand, predation-mediated viability selection against gigantism has been identified as one of the critical factors responsible for keeping organisms small (Blanckenhorn 2000), but there are other studies that support the idea that bigger is not always better (DiBattista *et al.* 2007; Carlson, Olsen & Vøllestad 2008). Predation selects against large size in several ways, for instance restricting activity levels and thus access to food and thereby growth (i.e. through behavioural phenotypic plasticity) (Sih 1982; Lima & Dill 1990) and by causing size-selective mortality (Werner & Gilliam 1984; Abrams & Rowe 1996). Also empirical studies have demonstrated that predation selects against large or fast-growing phenotypes (e.g. Lankford, Billerbeck & Conover 2001; Biro *et al.* 2004, 2006). It is noteworthy that gape-limited predation can select for an increase in foraging and growth in prey which then can reach a size refuge quickly (Williams 1966; Urban 2007). This has been found in many taxa including crustaceans (Black 1993), gastropods (Crowl & Covich 1990), fish (Magnhagen & Heibo 2004; Nakazawa *et al.* 2007) and amphibians (Urban 2008). Besides body size and growth, organisms can also modify their general morphology as a response to gape-limited predation, for example by developing protective spines in invertebrates or developing deeper bodies in fish (Parejko & Dodson 1991; Brönmark & Miner 1992).

In addition to predation, food supply is an obvious environmental factor affecting growth rates and body size during ontogeny (Clutton-Brock, Albon & Guinness 1985; Blanckenhorn 2000; Arendt & Reznick 2005). Therefore, variation in the levels of intraspecific competition, or more generally, access to food, is expected to affect optimal growth rates and body size both at the ontogenetic and genetic time-scales (for review, see Dmitriew 2011). While there are numerous studies focusing either on the evolutionary or plastic determinants of body size and growth with respect to variation in predation and resource availability (e.g. Lomolino 2005; Meiri, Cooper & Purvis 2008; Dmitriew 2011), less focus has been directed towards how local adaptation to a certain factor affects phenotypic plasticity expressed towards perceived variation in the same factor.

The nine-spined stickleback (*Pungitius pungitius*) occurs in a wide range of habitats (e.g. Bănărescu & Paepke 2001; Östlund-Nilsson, Mayer & Huntingford 2007). Our study system consists of ponds that are free of piscine predators and of marine populations that are sympatric to a number of predatory fish species. Stochasticity is likely to have a significant role in the temporal availability of resources in small ponds, and level of competition over resources is likely to be variable over the years, as we have observed large fluctuations in fish density throughout the years (Gábor Herczeg pers. obs.). In marine environments, resources are expected to be more stable spatially and temporally. A recent study showed that several pond populations in our system of nine-spined stickleback have evolved into giants when compared with coastal marine (or lake) populations, and both predation and competition have been implicated to have significant roles in the process (Herczeg, Gonda & Merilä 2009a). Further, it seems that the gigantism in pond nine-spined stickleback is mainly driven by fecundity selection acting on females (Herczeg, Gonda & Merilä 2010a). Group living has also been found to have a higher cost for pond than marine fish: when raised in groups instead of individual rearing, pond fish restrict their growth and develop relatively smaller brains, whereas no such difference has been found in marine fish (Gonda, Herczeg & Merilä 2009a; Herczeg, Gonda & Merilä 2009b). Pond fish are also bolder, more aggressive and explorative and have higher feeding activity than coastal marine populations (Herczeg, Gonda & Merilä 2009c; Herczeg & Välimäki 2011). Hence, nine-spined stickleback offers an excellent model to study local adaptation in phenotypic plasticity in body size as a response to varying predation pressure and resource availability.

The aim of this study was to examine how local selective pressures, predation and variability in resources/competition have shaped the expression of plasticity towards variation in the food supply and perceived predation risk using nine-spined stickleback as a model. Because females seem to drive body size evolution in the species (Herczeg, Gonda & Merilä 2010a; Herczeg *et al.* 2012), we were also interested to see whether there is divergence in plasticity between sexes. To achieve this, we studied reaction norms in size and growth in response to manipulated perceived predation risk and food supply in a factorial experiment using F1 laboratory generation of replicated high predation risk-adapted (coastal marine) and high intraspecific competition-adapted (pond) stickleback populations. More specifically, we predicted that (i) growth in stickleback from populations with high predation will be depressed by perceived predation risk to a larger extent than that of stickleback from populations without predation, (ii) growth in stickleback from populations with high intraspecific competition will be constrained by food shortage to a larger extent than that of stickleback from populations with low competition, and (iii) the treatments will have a more profound effect on female (the sex driving body size evolution in the species) stickleback growth than that of males.

Materials and methods

SAMPLING SITES

Adult nine-spined stickleback were collected from six sites (two pond and four coastal marine sites, Fig. 1) in the beginning of the breeding season of 2009 using minnow traps and seine nets. Three of the coastal sites were shallow marine bays (Helsinki, Nyköping and Kristineberg), and one was a coastal creek (Bölesviken) that was directly connected to the sea and was classified as a coastal marine population. Marine stickleback are sympatric to a large number of predatory and competitor fish species. The two ponds – Pyöreälampi (Finland) and Abbortjärnen (Sweden) – are small (surface area < 5 ha) and free of any sympatric fish species. Recently introduced small-sized whitefish (*Coregonus lavaretus*) might occur in Pyöreälampi in very low densities, but as whitefish are mostly planktivorous, we expect them to be only competitors, and not predators to the nine-spined stickleback (Kahilainen *et al*. 2004). Further, because of the low number of whitefish (we never caught one during several years' sampling), even the competition effect is likely to be negligible. As the ponds are small in size, they do not sustain a permanent population of piscivorous birds. Even though some birds can sporadically visit the ponds, the predation imposed by them most certainly does not exceed predation by birds in coastal marine areas. During the early life stages of nine-spined stickleback, cannibalism and predation by aquatic invertebrates are likely to be relevant in both habitat types. At any rate, the loss of antipredatory armour and the extreme longevity of pond sticklebacks are both hinting at relaxed predation (Herczeg, Gonda & Merilä 2009a; Herczeg, Turtiainen & Merilä 2010b). Adult fish were transported to the aquacultural facilities of the University of Helsinki and were kept in +17 °C and constant light to mimic reproductive conditions at these latitudes. Fish were fed with frozen bloodworms (Chironomidae*)* in excess until females reached reproductive condition.

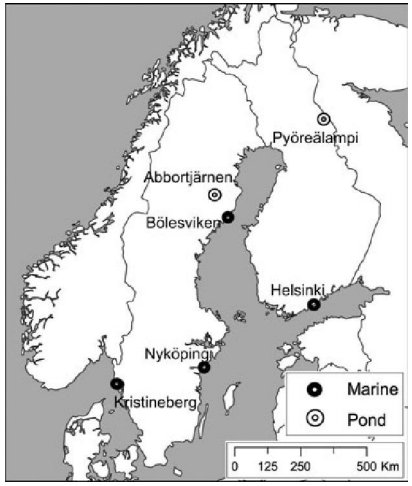


Fig. 1. Map of Fennoscandia showing the nine-spined stickleback populations used in the study.

BREEDING AND REARING

Between 1 July and 3 August, 2009, 6–9 artificial crosses per population were done (Abbortjärnen = 6, Bölesviken = 6, Helsinki = 8, Kristineberg = 10, Nyköping = 8, Pyöreälampi = 7). The freshly hatched fry were placed into individual 1·4 L containers of one of four Allentown Zebrafish Rack Systems (Aquaneering Inc., San Diego, CA, USA, hereafter 'rack'), aiming to an equal overall population/family/treatment representation. Individual containers were separated with white plastic panels to block visual contact among individual fish. One hundred containers were used in every rack, summing to 400 available containers. Mortality occurred mainly at early life stages, and 309 fish were analysed after 240 days of rearing. This means that a few families were not represented in every treatment combinations. However, given that we used family information only to correct for pseudoreplication, this should not cause any problem in the interpretation of our results. More details about the breeding and rearing procedures are given in Herczeg, Gonda & Merilä (2009b,c) and Herczeg & Välimäki (2011).

TREATMENTS

Fish were divided randomly and evenly into four treatments; two predation and two food-level treatments. For the predation treatment, every rack was fitted with a single external 150-L tank (four tanks in total) housing the predators in the predation treatment. Water (after being filtered by biological and mechanical filters) entered these additional tanks before it returned to the rack and to the individual containers. Two tanks were randomly designated as predator tanks and two as controls (no predator). In the predation treatment, we placed two 10- to 15-cm-long perch (*Perca fluviatilis*) – common predators of nine-spined stickleback (Koli *et al*. 1988) – into each of the two randomly chosen external tanks. We note that while perch is primarily a freshwater fish, it can be found in large numbers in the Baltic coastline because of the low salinity of the Baltic Sea (Ådjers *et al*. 2006), and it is probably one of the most abundant predatory fish species in freshwaters of Fennoscandia. In the two control racks, the extra tanks were filled up with water but no predator was introduced. Hence, the predator treatment corresponded to the presence/absence of olfactory cues from a common stickleback predator. Perch were fed with frozen bloodworms. For the food treatments, fish within families, predation treatments and racks were divided randomly into high and low food groups. Fish in the high food treatment were fed twice a day in excess, whereas fish in the low food treatment received food in excess only once in every 2 days. The photoperiod was set to 14 : 10 (light : dark), and the water temperature was held constant at 12 °C. Feeding was started with live brine shrimp (*Artemia salina*) nauplii, and after 80 days, the diet was gradually changed to frozen bloodworms. Because Baltic sampling sites are especially low in salinity, all rearing was performed in freshwater. Previous experiments on nine-spined stickleback in our system have shown that habitat-specific size patterns observed in common garden conditions closely resemble the differences observed in the wild, irrespective of the population of origin (Herczeg, Gonda & Merilä 2009a).

MEASUREMENTS

The fish were photographed three times at the age of 120, 180 and 240 days. All photographs were taken with a Nikon D60 digital camera (Nikon Corporation, Chiyoda, Tokyo, Japan) on a tripod under standardized conditions. A piece of millimetre paper was placed in every photograph for scaling. Standard length (from the tip of the snout to the end of the tail base) was measured from lateral pictures using software TPSDIG 2.15 (Rohlf 2006). At the end of the experiment, the stickleback were over-anesthetized with MS 222 (tricaine methanesulfonate) and stored frozen at −80 °C for further use. The sex of all the sticklebacks was verified by gonadal inspection.

STATISTICAL ANALYSES

Body size analyses was performed using a repeated measures general linear mixed model as implemented in PROC MIXED in SAS (Littell *et al*. 2006) with standard length as a dependent variable, food and predation treatments, habitat (marine or pond) and sex as fixed factors. Repeated measures at different ages were treated as a repeated measures factor, and we used both family nested in population and population nested in habitat as random factors to control for the non-independence of individuals within family, and populations within habitat. All two- and three-way interactions between the fixed factors and the single explanatory variables were included in the initial model. We used both visual inspection of the data structure and Akaike Information Criterion (AIC) (Littell, Rendergast & Natarajan 2000) to select the best fitting covariate structure for the analysis. The tested covariate structures were unstructured, auto regressive, Toeplitz, heterogenous Toeplitz and heterogenous first-order autoregressive. We chose the last one as it gave dearly the lowest AIC values. We applied backward stepwise model selection based on the $P < 0{\cdot}05$ criterion as there were several non-significant factor × covariate interactions in the initial model (see Engqvist 2005). We first removed non-significant three-way interactions in order of decreasing P-value and then moved to two-way interactions and finally to single effects. We did not remove any two-way interactions that were included in significant three-way interactions or single effects involved in any significant interactions. All analyses were performed by SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

The effect of perceived predation risk showed complex habitat- and sex dependence as indicated by the significant three-way interaction (Table 1). Marine fish and pond males developed smaller body size under perceived predation risk than in its absence, while the body size of female pond sticklebacks was not affected by the presence/absence of perch olfactory cues (Fig. 2). The effect of food manipulation was also habitat dependent (Table 1): the positive response to elevated food supply was stronger in pond than in marine fish (Fig. 3a). Growth in general (represented by the repeated measures factor) differed also between habitats, pond fish growing faster than marine fish (Fig. 3b).

The effect of food manipulation differed between sexes (Table 1), females showing stronger response to elevated food supply than males (Fig. 3c). Growth was also sex dependent (Table 1): females grew faster than males (Fig. 3d). We also found a significant predation treatment × food treatment interaction (Table 1). The positive effect of high food supply was only present in the absence of predation (Fig. 3e). Finally, growth was significantly faster in the high than in the low food treatment (Fig. 3f; Table 1).

Table 1. Summary of the repeated measures general linear mixed model of standard length of 120-, 180- and 240-day-old nine-spined stickleback

Effect	*ndf*	*ddf*	*F*	*P*
Habitat	1	4·06	4·2	0·108
Sex	1	273	33·7	**< 0·0001**
Predation	1	283	23·87	**< 0·0001**
Food	1	264	38·82	**< 0·0001**
Repeat	2	520	1476·3	**< 0·0001**
Habitat*sex	1	280	5·18	**0·024**
Habitat*food	1	271	5·63	**0·018**
Habitat*predation	1	282	1·21	0·280
Habitat*repeat	2	520	24·95	**< 0·0001**
Sex*food	1	278	5·90	**0·016**
Sex*predation	1	293	0·43	0·513
Sex*repeat	2	520	9·77	**< 0·0001**
Feed*repeat	2	520	3·80	**0·023**
Predation*repeat	2	518	0·04	0·960
Predation*food	1	269	4·46	**0·036**
Habitat*sex*predation	1	293	−5·99	**0·015**
Habitat*sex*food	1	285	0·41	0·525
Habitat*sex*repeat	2	518	0·22	0·802
Habitat*predation*food	1	267	0·55	0·460
Habitat*food*repeat	2	518	2·04	0·132
Habitat*predation*repeat	4	515	0·08	0·989
Sex*predation*food	1	280	1·03	0·311
Sex*predation*repeat	4	516	0·21	0·934
Sex*food*repeat	2	518	0·62	0·537
Predation*food*repeat	4	516	0·74	0·567

Non-significant effects (when not being part of a higher level significant interaction) are shown as seen at the one-by-one back-substitution to the final model. For further details, see Methods. Significant effects are given in bold font.

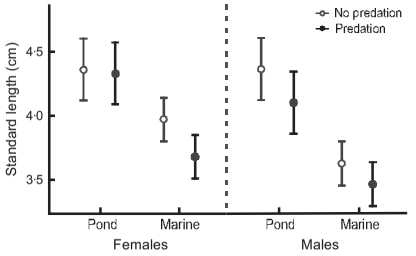


Fig. 2. Significant three-way interaction between habitat, sex and predation treatment in the repeated measures general linear mixed model of body size. Least squares means and standard errors are shown.

Discussion

The most salient finding of this study was that local adaptation to a certain environmental factor appears to involve the ability to express a higher degree of phenotypic plasticity induced by stimuli imitating variation in that certain environmental factor. We demonstrated that (i) predation-adapted

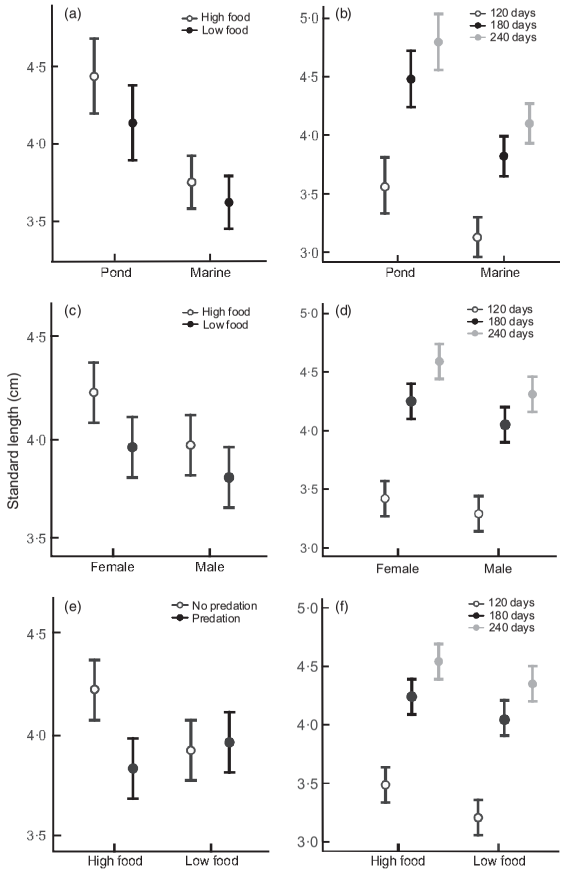


Fig. 3. Significant two-way interactions between habitat, sex and treatments in the repeated measures general linear mixed model of body size. (a) habitat × food treatment, (b) habitat × repeated measures factor (i.e. growth), (c) sex × food treatment, (d) sex × repeated measures factor, (e) food treatment × predation treatment, (f) food treatment × repeated measures factor. Least squares means and standard errors are shown.

marine stickleback reacted to the perceived predation risk by decreasing their growth to a larger extent than fish from pond populations and (ii) competition-adapted pond stickleback showed a greater increase in body size in the high food treatment than marine fish. Further, it seems that the body size evolution of nine-spined stickleback is driven by fecundity selection acting on females, and males are most probably only following females via correlated evolution (Herczeg, Gonda & Merilä 2010a). Results of this study indicate that the decreased response to perceived predation risk in ponds is driven by females which did not react to differences in perceived predation risk at all. Females showed stronger reaction to the food treatment than males too.

The fitness of an individual is determined by two main life-history components: survival and reproduction (Roff 1992; Stearns 1992). Predation is the most thoroughly studied, and perhaps also one of the most significant factors affecting individual survival probability in many organisms. Predator-induced plasticity is well known both in morphology (Parejko & Dodson 1991; Brönmark & Miner 1992; Relyea 2002; Gonda *et al.* 2009b; Frommen *et al.* 2011) and behaviour (Laurila, Pakkasmaa & Merilä 2006; Bell & Sih 2007; Chiba, Arnott & Conover 2007, Robinson, Januszkiewicz & Koblitz 2007). The threat of predation can also influence body size and growth rate in many ways. In general, animals may restrict their movement and maximize the time spent in cover at the cost of time spent foraging, which will lead to a lower energy intake and consequently slower growth rate and reduced body size (for review, see Dmitriew 2011). There are exceptions though: for instance,

decreased activity can also make fish large and fat if food is otherwise easily available (Johansson & Andersson 2009), fish might try to escape gape-limited predation by growing larger fast (Lundvall *et al.* 1999; Urban 2007; Bell *et al.* 2011), and thinning of the prey population by predation might also result in higher per capita food availability and therefore in increased growth rates and larger body size (Werner & Gilliam 1984; Ernsting *et al.* 1999; : Grether *et al.* 2001; Arendt & Reznick 2005). It is noteworthy that perceived predation risk increases growth in three-spined stickleback (*Gasterosteus aculeatus*) (Frommen *et al.* 2010; Bell *et al.* 2011), while we found the opposite pattern in nine-spined stickleback. One possible reason for the difference between these two closely related species is that while the complex of the pelvic girdle, pelvic spines, lateral plates and dorsal spines in three-spined stickleback forms a strong functional unit (Reimchen 1983), which restricts gape-limited predators efficiently, the armour of nine-spined stickleback is weaker and provides less protection (Hoogland, Morris & Tinbergen 1957). Therefore, faster growth might allow three-spined stickleback to escape predation by reaching a safe size (e.g. Moodie 1972a,b; Reimchen 1991), while such effect is less likely for the nine-spined stickleback.

We found a habitat- and sex-specific response to perceived predation risk: the expected size deficit was only absent in pond females. We do not have exact knowledge on the mechanism underlying the observed size difference between predation treatments. One obvious explanation could be that fish simply change their behaviour and limit their feeding to avoid being conspicuous. However, in a previous paper published from the same experiment, we found that predation treatment did not affect the probability of initiating feeding (Herczeg & Välimäki 2011). It is likely that smaller size of fish observed in the predation treatment is because of some unmeasured behavioural aspects of feeding (i.e. time spent feeding or amount of food eaten). Alternatively, predation-adapted fish might invest more energy into physiological performance instead of growth when a predator is present, to develop better escape abilities. It could also be that decreased growth is a by-product of elevated metabolism in response to stress in these fish, representing maladaptive plasticity (*sensu* Ghalambor *et al.* 2007). For instance, it has been shown that three-spined stickleback originating from high predation populations have elevated ventilation rates under the threat of predation compared with low predation population sticklebacks (Bell, Henderson & Huntingford 2010). At any rate, only pond females were clearly unaffected by the olfactory cues of a common stickleback predator. Interestingly, they are the sex that is suspected to drive the evolution of gigantism observed in ponds (Herczeg, Gonda & Merilä 2010a; Herczeg *et al.* 2012). In theory, one could claim that not decreasing growth under predation risk can be an adaptive response to reaching a size refuge (e.g. Urban 2007). However, in our case, pond fish have evolved under the lack of fish predation, and hence, their lack of response cannot be an adaptation to predation by fish, but rather an adaptation to the lack of predation.

When access to food is unlimited, animals typically grow faster and to a larger size than when food resources are limited (Lind, Persbo & Johansson 2008; Dmitriew 2011). We found the predicted pattern: fish in the high food treatment grew faster and larger. In line with our expectations, pond fish showed stronger responses to the food treatment than marine fish. In pond populations where predation and interspecific competition are relaxed, nine-spined stickleback have evolved giant sizes (Herczeg, Gonda & Merilä 2009a). We assume that food availability and intraspecific competition are major selective factors in ponds based on the following points: (i) resource levels are expected to be temporally unstable because of the small size of the isolated water bodies (< 5 ha surface area), (ii) pond fish have a longer period of intensive growth, have higher unit volume per unit time growth rate and reach larger size than marine fish (Herczeg *et al.* 2012), and (iii) pond fish are more aggressive than marine fish and also suffer great developmental costs from group living while marine fish do not (Gonda, Herczeg & Merilä 2009a; Herczeg, Gonda & Merilä 2009b,c; Herczeg & Välimäki 2011). Therefore, assuming that pond stickleback are adapted to a high competition/resource-limited environment with their probably higher-than-average energy needs, it is not surprising that they utilized the extra food much better than the marine fish. It is also noteworthy that we found that the positive effect of extra food was mainly apparent when the predation risk was absent. This might reflect to the fact that there is a foraging activity – survival trade-off under predation risk in the wild (Lima & Dill 1990). This result shows that the presence of perceived predation risk alone can result in submaximal growth.

We recently found strong support for hyperallometry in sexual size dimorphism in nine-spined stickleback, with females being the larger sex (Herczeg, Gonda & Merilä 2010a). An interesting aspect of allometry in sexual size dimorphism is that the gender with the larger size variation is assumed to drive the body size evolution in the system, while the other sex follows it because of correlative selection (Fairbairn 1997), suggesting that female stickleback drive body size evolution in our system. Body size is positively correlated with reproductive output in nine-spined stickleback females (Heins, Johnson & Baker 2003; Herczeg, Gonda & Merilä 2010a), and thus, fecundity selection on female reproductive output has been suggested to be the main factor driving the evolution of gigantism in nine-spined stickleback (Herczeg, Gonda & Merilä 2010a). Hence, we expected that females would be the dominant sex in shaping patterns of habitat divergence in the extent of plasticity induced by our treatments. Our results show that this was indeed the case in our system. Females (especially from ponds) utilized the surplus energy in the high food treatment better than the males. Furthermore, all groups, except the pond females, responded to the predation treatment by developing smaller bodies. Apparently, pond females are trying to maximize their size even under the threat of predation. This might be implicative of the importance of fecundity selection in the ponds.

The relationship between local adaptation and phenotypic plasticity is complex and highly discussed (e.g. de Jong 2005; Pigliucci, Murren & Schlichting 2006, Crispo 2008). Phenotypic plasticity itself can be seen as either a quantitative trait under selection, or a developmental process facilitating adaptive divergence, or even speciation (de Jong 2005; Pigliucci, Murren & Schlichting 2006; Pfennig *et al*. 2010). Environmental sensitivity of a trait can either increase or decrease during the course of evolution (e.g. Pfennig *et al*. 2010). Intuitively, one would expect the situation to change with the time since an environmental change has taken place (e.g. after the invasion of a new environment) and the within/between generation variability of the environmental factor inducing phenotypic plasticity and genetic adaptation. For instance, time since environmental change might correlate negatively with the magnitude of phenotypic plasticity if the relevant environmental factors were stable (genetic assimilation towards an extreme phenotype provided by plasticity, Waddington 1953; Crispo 2007). Alternatively, the correlation might be positive if the environmental factors show high variation (phenotypic plasticity itself is adaptive, Richter-Boix, Llorente & Montori 2006, Lind & Johansson 2007; Kishida, Trussell & Nishimura 2007). While our data set is not adequate to test these opposing models/hypotheses directly, the patterns we report here support the idea that genetic adaptation to a certain environmental factor includes increased ontogenetic sensitivity towards stimuli containing information about that factor, which suggests that phenotypic plasticity evolves like a quantitative trait.

Interestingly, when one looks at the population-level patterns (Fig. S1, Supporting information), it seems evident that while the 'true' marine populations are showing the described habitat-specific patterns (smaller size in predation treatment and less response in the food treatment), the population (Bölesviken) that was collected from a creek directly connected to sea responded to the treatment mainly like pond fish. This was surprising as this population shares the morphological features of a marine fish (small body size, large number of plates and long pelvic spines) (Herczeg, Gonda & Merilä 2009a; Herczeg, Turtiainen and Merilä 2010). For further inspection, we reran all analysis excluding this population, but it did not change the results qualitatively. It appears that selective pressures in this creek population might differ from the ones that the 'true' marine populations are exposed to. It has been reported earlier that there can be strong morphological variation in a very small geographical scale between connected nine-spined stickleback populations (Ziuganov & Zotin 1995), and our case further emphasizes how habitat characterization must be done with utmost rigour.

In summary, we found that nine-spined stickleback populations from sites with high predation risk expressed a higher degree of phenotypic plasticity in body size in response to manipulated perceived predation risk as compared to their conspecifics from sites with low predation risk. Furthermore, individuals originating from populations with high intraspecific competition expressed higher levels of body size phenotypic plasticity in response to manipulated resource levels as compared to their conspecifics from populations with lower intraspecific competition. These habitat-dependent, genetically based differences in phenotypic plasticity trends strongly imply that the pattern is a result of natural selection (e.g. Clarke 1975; Endler 1986; McGuigan, Chenoweth & Blows 2005). In addition, we found that females, which are expected to drive the body size evolution in this species, were the dominant group shaping the patterns. We note that the number of populations in our study was relatively low, but the within-habitat replicates are geographically separated by several hundreds of kilometres, and the ponds are also genetically highly isolated both from each other as well as the marine populations (Shikano *et al*. 2010) making the replicates truly independent. These results support the argument that individuals adapted to selection pressures imposed by certain environmental factors are also ontogenetically more sensitive to those environmental factors than are the individuals from populations under weaker selection. We demonstrated this phenomenon at two levels: (i) among (i.e. habitat type) and within (i.e. sex) populations.

Acknowledgements

Göran Englund, Jacquelin DeFaveri, Jenni Hämäläinen, Lotta Kvarnemo, Tuomas Leinonen, Pia Rännänen and Pirkko Siikamäki helped to obtain the samples. Mirva Turtiainen, Linda Uoti, Maarit Haataja, Nurul Izza Ab Ghani, Riikka Kaartinen, Joona Lehtomäki and Marika Lilja helped with fish maintenance. We thank Anssi Laurila, Tuomas Leinonen and Juha Merilä for commenting earlier versions of this manuscript and Amber Teacher for correcting the English. Thanks also to Yukinori Shimada for discussion and help with the practicalities and to two anonymous reviewers for their constructive comments that improved this paper. Our work was supported by Academy of Finland (GH #128716; grants to Juha Merilä #134728 & #129662) and Finnish Graduate School in Population Genetics (KV). The experiment was conducted under the licence of the Finnish National Animal Experiment Board (Licence number STH223A).

References

Abrams, P.A. & Rowe, L. (1996) The effects of predation on the age and size of maturity of prey. *Evolution*, **50**, 1052–1061.

Ådjers, K., Appelberg, M., Eschbaum, R., Lappalainen, A., Minde, A., Repecka, R. & Thoresson, G. (2006) Trends in coastal fish stocks of the Baltic Sea. *Boreal Environment Research*, **11**, 13–25.

Andersson, M. (1994) *Sexual Selection*. Princeton University Press, Princeton, NJ.

Arendt, J.D. & Reznick, D.N. (2005) Evolution of juvenile growth rates in female guppies (*Poecilia reticulata*): predator regime or resource level? *Proceedings of the Royal Society B*, **272**, 333–337.

Auld, J.R., Agrawal, A.A. & Relyea, R.A. (2010) Re-evaluating the costs and limits of adaptive phenotypic plasticity. *Proceedings of the Royal Society B*, **277**, 503–511.

Bănărescu, P.M. & Paepke, H.-J. (2001) *The Freshwater Fishes of Europe*, Vol. 5/III. AULA-Verlag, Wiebelsheim.

Bell, A.M., Henderson, L. & Huntingford, F.A. (2010) Behavioral and respiratory responses to stressors in multiple populations of three-spined sticklebacks that differ in predation pressure. *Journal of Comparative Physiology. B*, **180**, 211–220.

Bell, A.M. & Sih, A. (2007) Exposure to predation generates personality in threespined sticklebacks (*Gasterosteus aculeatus*). *Ecology Letters*, **10**, 828–834.

Bell, A.M., Dingemanse, N.J., Hankison, S.J., Langenhof, M.B.W. & Rollins, K. (2011) Early exposure to nonlethal predation risk by size-selective predators increases somatic growth and decreases size at adulthood in threespined sticklebacks. *Journal of Evolutionary Biology*, **24**, 943–953.

Biro, P.A., Abrahams, M.V., Post, J.R. & Parkinson, E.A. (2004) Predators select against high growth rates and risk-taking behaviour in domestic trout populations. *Proceedings of the Royal Society B*, **271**, 2233–2237.

Biro, P.A., Abrahams, M.V., Post, J.R. & Parkinson, E.A. (2006) Behavioural trade-offs between growth and mortality explain evolution of submaximal growth rates. *Journal of Animal Ecology*, **75**, 1165–1171.

Black, A.R. (1993) Predator-induced phenotypic plasticity in *Daphnia pulex*: life history and morphological responses to Notonecta and Chaoborus. *Limnology and Oceanography*, **38**, 986–996.

Blanckenhorn, W.U. (2000) The evolution of body size: what keeps organisms small? *Quarterly Review of Biology*, **75**, 385–407.

Brönmark, C. & Miner, J.G. (1992) Predator-induced phenotypical change in body morphology in crucian carp. *Science*, **258**, 1348–1350.

Carlson, S.M., Olsen, E.M. & Vøllestad, L.A. (2008) Seasonal mortality and the effect of body size: a review and an empirical test using individual data on brown trout. *Functional Ecology*, **22**, 663–673.

Chiba, S., Arnott, S.A. & Conover, D.O. (2007) Coevolution of foraging behavior with intrinsic growth rate: risk-taking in naturally and artificially selected growth genotypes of *Menidia menidia*. *Oecologia*, **154**, 237–246.

Clarke, B.C. (1975) The contribution of ecological genetics to evolutionary theory: detecting the direct effects of natural selection on particularly polymorphic loci. *Genetics*, **79**, 101–113.

Clutton-Brock, T.H., Guiness, F.E. & Albon, S.D. (1982) *Red deer: behaviour and ecology of two sexes*. University of Chicago Press, Chicago, IL.

Clutton-Brock, T.H., Albon, S.D. & Guinness, F.E. (1985) Parental investment and sex differences in juvenile mortality in birds and mammals. *Nature*, **313**, 131–133.

Conover, D.O., Duffy, T.A. & Hice, L.A. (2009) The covariance between genetic and environmental influences across ecological gradients: reassessing the evolutionary significance of countergradient and cogradient variation. *Annals of the New York Academy of Sciences*, **1168**, 100–129.

Conover, D.O. & Schultz, E.T. (1995) Phenotypic similarity and the evolutionary significance of countergradient variation. *Trends in Ecology and Evolution*, **10**, 248–252.

Crispo, E. (2007) The Baldwin effect and genetic assimilation: revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. *Evolution*, **61**, 2469–2479.

Crispo, E. (2008) Modifying effects of phenotypic plasticity on interactions among natural selection, adaptation and gene flow. *Journal of Evolutionary Biology*, **21**, 1460–1469.

Crowl, T.A. & Covich, A.P. (1990) Predator-induced lifehistory shifts in a freshwater snail. *Science*, **247**, 949–951.

DeWitt, T.J. & Scheiner, S.M. (2004) *Phenotypic Plasticity: Functional and Conceptual Approaches*. Oxford University Press, Oxford UK.

DeWitt, T., Sih, A. & Wilson, D.S. (1998) Costs and limits of phenotypic plasticity. *Trends in Ecology & Evolution*, **13**, 77–81.

DiBattista, J.D., Feldheim, K.A., Gruber, S.H. & Hendry, A.P. (2007) When bigger is not better: selection against large size, high condition and fast growth in juvenile lemon sharks. *Journal of Evolutionary Biology*, **20**, 201–212.

Dmitriew, C.M. (2011) The evolution of growth trajectories: what limits growth rate? *Biological Reviews*, **86**, 97–116.

Endler, J.A. (1986) *Natural Selection in the Wild*. Princeton University Press, Princeton, NJ.

Engqvist, L. (2005) The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Animal Behaviour*, **70**, 967–971.

Emande, B. & Dieckmann, U. (2004) The evolution of phenotypic plasticity in spatially structured environments: implications of intraspecific competition, plasticity costs and environmental characteristics. *Journal of Evolutionary Biology*, **17**, 613–628.

Emsting, G., Brandjes, G.J., Block, W. & Isaaks, A.A. (1999) Life-history consequences of predation for a subantarctic beetle: evaluating the contribution of direct and indirect effects. *Journal of Animal Ecology*, **68**, 741–752.

Fairbairn, D.J. (1997) Allometry for sexual size dimorphism: patterns and process in the coevolution of body size in males and females. *Annual Review of Ecology and Systematics*, **28**, 659–687.

Frommen, J.G., Herder, F., Engqvist, L., Mehlis, M., Bakker, T.C.M., Schwarzer, J. & Thünken, T. (2011) Costly plastic morphological responses to predator specific odour cues in three-spined sticklebacks (*Gasterosteus aculeatus*). *Evolutionary Ecology*, **25**, 641–656.

Fusco, G. & Minelli, M. (2010) Phenotypic plasticity in development and evolution: facts and concepts. *Philosophical Transactions of the Royal Society B*, **365**, 547–556.

Ghalambor, C.K., McKay, J.K., Carroll, S.P. & Reznick, D.N. (2007) Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology*, **21**, 394–407.

Gomulkiewicz, R. & Kirkpatrick, M. (1992) Quantitative genetics and the evolution of reaction norms. *Evolution*, **46**, 390–411.

Gonda, A., Herczeg, G. & Merilä, J. (2009a) Habitat-dependent and -independent plastic responses to social environment in the ninespine stickleback (*Pungitius pungitius*) brain. *Proceedings of the Royal Society B*, **276**, 2085–2092.

Gonda, A., Trokovic, N., Herczeg, G., Laurila, A. & Merilä, J. (2009b) Predation- and competition-mediated brain plasticity in *Rana temporaria* tadpoles. *Journal of Evolutionary Biology*, **23**, 2300–2308.

Grether, G.F., Millie, D.F., Bryant, M.J., Reznick, D.N. & Mayea, W. (2001) Rain forest canopy cover, resource availability, and life history evolution in guppies. *Ecology*, **82**, 1546–1559.

Heins, D.C., Johnson, J.M. & Baker, J.A. (2003) Reproductive ecology of the nine-spined stickleback from south-central Alaska. *Journal of Fish Biology*, **63**, 1131–1143.

Herczeg, G., Gonda, A. & Merilä, J. (2009a) Evolution of gigantism in nine-spined stickleback. *Evolution*, **63**, 3190–3200.

Herczeg, G., Gonda, A. & Merilä, J. (2009b) The social cost of shoaling covaries with predation risk in nine-spined stickleback (*Pungitius pungitius*) populations. *Animal Behaviour*, **77**, 575–580.

Herczeg, G., Gonda, A. & Merilä, J. (2009c) Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *Journal of Evolutionary Biology*, **22**, 544–552.

Herczeg, G., Gonda, A. & Merilä, J. (2010a) Rensch's rule inverted – female-driven gigantism in nine-spined stickleback (*Pungitius pungitius*). *Journal of Animal Ecology*, **79**, 581–588.

Herczeg, G., Turtiainen, M. & Merilä, J. (2010) Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*). *Biological Journal of the Linnean Society*, **101**, 403–416.

Herczeg, G. & Välimäki, K. (2011) Intraspecific variation in behaviour: effects of evolutionary history, ontogenetic experience and sex. *Journal of Evolutionary Biology*, **24**, 2434–2444.

Herczeg, G., Gonda, A., Kuparinen, A. & Merilä, J. (2012) Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? *Evolutionary Ecology*, **26**, 109–122.

Hoogland, R., Morris, D. & Tinbergen, N. (1957) The spines of sticklebacks (*Gasterosteus* and *Pygosteus*) as means of defence against predators (*Perca* and *Esox*). *Behaviour*, **10**, 205–236.

Johansson, F. & Andersson, J. (2009) Scared fish get lazy, and lazy fish get fat. *Journal of Animal Ecology*, **78**, 772–777.

de Jong, G. (2005) Evolution of phenotypic plasticity: patterns of plasticity and the emergence of ecotypes. *New Phytologist*, **106**, 101–117.

Kahilainen, K., Malinen, T., Tuomaala, A. & Lehtonen, H. (2004) Diel and seasonal habitat and food segregation of three sympatric *Coregonus lavaretus* forms in a subarctic lake. *Journal of Fish Biology*, **64**, 418–434.

Kawecki, T.J. & Ebert, D. (2004) Conceptual issues in local adaptation. *Ecology Letters*, **7**, 1225–1241.

Kishida, O., Trussell, G.C. & Nishimura, K. (2007) Geographic variation in a predator-induced defense and its genetic basis. *Ecology*, **88**, 1948–1954.

Koli, L., Rask, M., Viljanen, M. & Aro, E. (1988) The diet of perch, *Perca fluviatilis* L., at Tvärminne, Northern Baltic Sea, and a comparison with two lakes. *Aqua Fennica*, **18**, 185–191.

Lankford, T.E., Billerbeck, J.M. & Conover, D.O. (2001) Evolution of intrinsic growth and energy acquisition rates. II. Trade-offs with vulnerability to predation in Menidia menidia. *Evolution*, **55**, 1873–1881.

Laurila, A., Pakkasmaa, S. & Merilä, J. (2006) Population divergence in growth rate and antipredator defences in *Rana arvalis*. *Oecologia*, **147**, 585–595.

Lima, S.L. & Dill, L.M. (1990) Behavioural decisions made under the risk of predation – a review and prospectus. *Canadian Journal of Zoology*, **68**, 619–640.

Lind, M.I. & Johansson, F. (2007) The degree of adaptive phenotypic plasticity is correlated with the spatial environmental heterogeneity experienced by island populations of *Rana temporaria*. *Journal of Evolutionary Biology*, **20**, 1288–1297.

Lind, M.I., Persbo, F. & Johansson, F. (2008) Pool desiccation and developmental thresholds in the common frog, *Rana temporaria*. *Proceedings of the Royal Society B*, **275**, 1073–1080.

Littell, R.C., Rendergast, J. & Natarajan, R. (2000) Modeling covariance structure in the analysis of repeated measures data. *Statistics in Medicine*, **19**, 1793–1819.

Littell, R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D. & Schabenberger, O. (2006) *SAS for Mixed Models*, 2nd edn. SAS Institute Inc, Cary, NC.

Lomolino, M.V. (2005) Body size evolution in insular vertebrates: generality of the island rule. *Journal of Biogeography*, **32**, 1683–1699.
Lundvall, D., Svanbäck, R., Persson, L. & Byström, P. (1999) Size-dependent predation in piscivores: interactions between predator foraging and prey avoidance abilities. *Canadian Journal of Fisheries and Aquatic Sciences*, **56**, 1285–1292.
Magnhagen, C. & Heibo, E. (2004) Growth in length and in body depth in young-of-the-year perch with different predation risk. *Journal of Fish Biology*, **64**, 612–624.
Masel, J., King, O.D. & Maughan, H. (2007) The loss of adaptive plasticity during long periods of environmental stasis. *American Naturalist*, **169**, 38–46.
McGuigan, K.S., Chenoweth, F. & Blows, M.W. (2005) Phenotypic divergence along lines of genetic variance. *American Naturalist*, **165**, 32–43.
Meiri, S., Cooper, N. & Purvis, A. (2008) The island rule: made to be broken? *Proceedings of the Royal Society B*, **275**, 141–148.
Moodie, G.E.E. (1972a) Predation, natural selection and adaptation in an unusual threespine stickleback. *Heredity*, **28**, 155–167.
Moodie, G.E.E. (1972b) Morphology, life history and ecology of an unusual stickleback (*Gasterosteus aculeatus*) in the Queen Charlotte Islands. *Canadian Journal of Zoology*, **50**, 721–738.
Moran, N.A. (1992) The evolutionary maintenance of alternative phenotypes. *American Naturalist*, **139**, 971–989.
Nakazawa, T., Ishida, N., Kato, M. & Yamamura, N. (2007) Larger body size with higher predation rate. *Ecology of Freshwater Fish*, **16**, 362–372.
Östlund-Nilsson, S., Mayer, I. & Huntingford, F.A. (2007) *Biology of the Three-spined Stickleback*. CRC Press, Boca Raton, FL.
Parejko, K. & Dodson, S.I. (1991) The evolutionary ecology of an antipredator reaction norm: *Daphnia pulex* and *Chaoborus americanus*. *Evolution*, **45**, 1665–1674.
Peters, R.H. (1983) *The Ecological Implications of Body Size*. Cambridge University Press, Cambridge.
Pfennig, D.W., Wund, M.A., Snell-Rood, E.C., Cruickshank, T., Schlichting, C.D. & Moczek, A.P. (2010) Phenotypic plasticity's impact on diversification and speciation. *Trends in Ecology and Evolution*, **25**, 459–467.
Pigliucci, M., Murren, C.J. & Schlichting, C.D. (2006) Phenotypic plasticity and evolution by genetic assimilation. *Journal of Experimental Biology*, **209**, 2362–2367.
Reimchen, T.E. (1983) Structural relationships between spines and lateral plates in threespine stickleback (*Gasterosteus aculeatus*). *Evolution*, **37**, 931–946.
Reimchen, T.E. (1991) Trout Foraging Failures and the Evolution of Body Size in Stickleback. *Copeia*, **1991**, 1098–1104.
Relyea, R.A. (2002) Competitor-induced plasticity in tadpoles: consequences, cues, and connections to predator-induced plasticity. *Ecological Monographs*, **72**, 523–540.
Richter-Boix, A., Llorente, G.A. & Montori, A. (2006) A comparative analysis of the adaptive developmental plasticity hypothesis in six Mediterranean anuran species along a pond permanency gradient. *Evolutionary Ecology Research*, **8**, 1139–1154.
Robinson, B.W., Januszkiewicz, A.J. & Koblitz, J.C. (2007) Survival benefits and divergence of predator-induced behavior between pumpkinseed sunfish ecomorphs. *Behavioral Ecology*, **19**, 263–271.
Roff, D.A. (1992) *The Evolution of Life Histories: Theory and Analysis*. Chapman and Hall, New York, NY.
Rohlf, F.J. (2006) TPS software series. Distributed by the author. Stony Brook, Department of Ecology and Evolution. State University of New York, NY.
Shikano, T., Shimada, Y., Herczeg, G. & Merilä, J. (2010) History vs. habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. *Molecular Ecology*, **19**, 1147–1161.
Shine, R. (1988) The evolution of large body size in females: a critique of Darwin's fecundity advantage model. *American Naturalist*, **131**, 124–131.
Shine, R. (1989) Ecological causes for the evolution of sexual size dimorphism: a review of the evidence. *Quarterly Review of Biology*, **64**, 419–461.
Sih, A. (1982) Foraging strategies and the avoidance of predation by an aquatic insect, *Notonecta hoffmani*. *Ecology*, **63**, 786–796.
Stearns, S.C. (1992) *The Evolution of Life Histories*. Oxford University Press, Oxford, U.K.
Urban, M.C. (2007) The growth-predation risk trade-off under a growing gape-limited predation threat. *Ecology*, **88**, 2587–2597.
Urban, M.C. (2008) Salamander evolution across a latitudinal cline in gape-limited predation risk. *Oikos*, **117**, 1037–1049.
Van Buskirk, J. & Steiner, U.K. (2009) The fitness costs of developmental canalization and plasticity. *Journal of Evolutionary Biology*, **22**, 852–860.
Via, S. & Lande, R. (1985) Genotype–environment interaction and the evolution of phenotypic plasticity. *Evolution*, **39**, 505–522.
Via, S., Gomulkiewicz, R., de Jong, G., Scheiner, S.M., Schlichting, C.D. & Vantienderen, P.H. (1995) Adaptive phenotypic plasticity: consensus and controversy. *Trends in Ecology and Evolution*, **10**, 212–217.
Waddington, C.H. (1953) The "Baldwin effect," "genetic assimilation" and "homeostasis". *Evolution*, **7**, 386–387.
Werner, E.E. & Gilliam, J.F. (1984) The ontogenetic niche and species interactions in size structured populations. *Annual Review of Ecology and Systematics*, **15**, 393–425.
West-Eberhard, M.J. (2003) *Developmental Plasticity and Evolution*. Oxford University Press, Oxford.
Williams, G.C. (1966) Natural selection, the cost of reproduction and a refinement of Lack's principle. *American Naturalist*, **100**, 687–690.
Wooton, R.J. (1979) Energy cost of egg production and environmental determinants of fecundity in teleost fishes. *Symposia of the Zoological Society of London*, **44**, 133–159.
Ziuganov, V.V. & Zotin, A.A. (1995) Pelvic girdle polymorphism and reproductive barriers in the ninespine stickleback *Pungitius pungitius* (L.) from northwest Russia. *Behaviour*, **132**, 1095–1105.

Received 12 May 2011; accepted 28 January 2012
Handling Editor: Shai Meiri

Supporting information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. A population level presentation of the significant habitat effects in our repeated measures general linear mixed model of nine-spined stickleback body size.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2012, **107**, 854–866. With 5 figures

Morphological anti-predator defences in the nine-spined stickleback: constitutive, induced or both?

KAISA VÄLIMÄKI*, GÁBOR HERCZEG† and JUHA MERILÄ

Ecological Genetics Research Unit, University Helsinki, PO Box 65, FI-00014 Helsinki, Finland

Received 23 May 2012; revised 20 June 2012; accepted for publication 20 June 2012

Environmental differences among populations are expected to lead to local adaptation, while spatial or temporal environmental variation within a population will favour evolution of phenotypic plasticity. As plasticity itself can be under selection, locally adapted populations can vary in levels of plasticity. Nine-spined stickleback (*Pungitius pungitius*) originating from isolated ponds (low piscine predation risk, high competition) vs. lake and marine populations (high piscine predation risk, low competition) are known to be morphologically adapted to their respective environments. However, nothing is known about their ability to express phenotypic plasticity in morphology in response to perceived predation risk or food availability/competition. We studied predator-induced phenotypic plasticity in body shape and armour of marine and pond nine-spined stickleback in a factorial common garden experiment with two predator treatments (present vs. absent) and two feeding regimes (low vs. high). The predation treatment did not induce any morphological shifts in fish from either habitat or food regime. However, strong habitat-dependent differences between populations as well as strong sexual dimorphism in both body shape and armour were found. The lack of predator-induced plasticity in development of the defence traits (viz. body armour and body depth) suggests that morphological anti-predator traits in nine-spined stickleback are strictly constitutive, rather than inducible. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **107**, 854–866.

ADDITIONAL KEYWORDS: adaptation – armour – body shape – common garden – phenotypic plasticity – predation – Pungitius pungitius.

INTRODUCTION

One of the key aims in evolutionary biology is to understand the origin and maintenance of genetic and phenotypic variation. Differences in local environmental conditions can impose divergent selective pressures, which can result in phenotypic divergence among local populations of the same species. This divergence can occur when populations become locally adapted to prevailing conditions through evolutionary response (Kawecki & Ebert, 2004). Alternatively, populations can diverge as a result of phenotypic plasticity (e.g. West-Eberhard, 2003). Whether populations respond to local selection pressures by genetic or plastic responses is thought to depend on the relative costs and benefits of the focal traits (DeWitt, Sih & Wilson, 1998; Richards *et al.*, 2006; Van Buskirk & Steiner, 2009).

Predation is one of the most important biotic factors affecting life history traits and population dynamics, and it has an important role in influencing morphological phenotypic variation within and among populations (Roff, 1992; Nosil & Crespi, 2006). For instance, a number of species have evolved protective morphological structures to avoid predation (Tollrian & Harvell, 1999). As many of those protective structures are costly to produce and/or maintain, they might be maladaptive in situations in which predation pressure is low (Harvell, 1990). Hence, when there is either temporal or spatial variation in predation pressure, the ability to express induced rather than constitutive defences might be beneficial to individuals. There are several examples of plasticity in morphological traits induced by predation (e.g. Stemberger & Gilbert, 1984; McCollum & Van Buskirk, 1996; Tollrian & Harvell, 1999; Johansson, 2002; Laforsch, 2004; Teplitsky *et al.*, 2005; Vaughn, 2007). Within fish, a well-known study of crucian carps (*Carassius carassius*) demonstrated that fish reared under perceived threat of predation from pike (*Esox lucius*) developed deeper bodies (Brönmark & Miner, 1992). Later it was shown that possession of a deeper body made individuals more difficult to handle for gape-limited pikes (Nilsson, Brönmark & Pettersson, 1995) and improved escape performance of the crucian carps (Domenici *et al.*, 2008), thus reducing the risk of predation. It has also been shown that predation risk can affect development of protective structures (armour traits) in fish: dorsal spines in pumpkinseed fish, *Lepomis gibbosus*, were longer when predators were present (Januszkiewicz & Robinson, 2007). Furthermore, an interplay between predator-induced plasticity and nourishment is also possible: in general, individuals with low body condition are able to invest less in induced defences (Noonburg & Nisbet, 2005; Teplitsky *et al.*, 2005; Chivers *et al.*, 2007), but an increased investment in protection while facing food stress has also been documented (e.g. Pauwels, Stoks & de Meester, 2010).

While predator-induced plasticity can be beneficial to individuals under conditions where predation pressure varies, less is known about what happens to induced defences when predation risk ceases to exist. It is possible that plastic anti-predatory traits can be retained in the population after threat of predation is relaxed. If the plastic traits are not expressed, selection cannot work directly on traits unless there is cost of maintaining the hidden trait or its plasticity (Lahti *et al.*, 2009). In a study of the Hokkaidō frog (*Rana pirica*), tadpoles from mainland populations commonly express protective bulky body shape in the vicinity of predatory salamanders. However, in island populations that are free of predatory salamanders, the tadpoles have lost their morphological responses to predators (Kishida, Trussel & Nishimura, 2007). Similarly, threat of predation from three-spined stickleback (*Gasterosteus aculeatus*) induced a stronger morphological response in common frog (*R. temporaria*) tadpoles originating from populations sympatric with stickleback than from populations lacking stickleback (Laurila, Pakkasmaa & Merilä, 2006). However, studies focusing on geographic variation in the expression of predator-induced morphological defences are as yet scarce.

Nine-spined stickleback (*Pungitius pungitius*) are small teleost fish that have a wide distribution throughout the northern hemisphere (Östlund-Nilsson, Mayer & Huntingford, 2007). The wide diversity of habitats they occupy offers excellent opportunities to study adaptive divergence in response to variation in biotic environment. In the related three-spined stickleback, body armour and spines offer effective means of protection against gape-limited predators (Reimchen, 1983). Although plates and spines are smaller and weaker in nine-spined stickleback, they do offer protection against predators (Hoogland, Morris & Tinbergen, 1957). Studies carried out on marine, lake and isolated pond populations of nine-spined stickleback have shown that there is a habitat-dependent population divergence in body shape and body armour (Ziuganov & Zotin, 1995; Herczeg, Turtiainen & Merilä, 2010; Mobley *et al.*, 2011). In general, populations living in environments free of piscine predation have reduced or absent pelvic spines and girdle and fewer lateral plates than marine or lake populations that are sympatric to a number of predatory fish species. Furthermore, fish from pond populations have also been found to have deeper bodies and shorter caudal peduncles than those from marine populations (Herczeg *et al.*, 2010). Studies in the same system have also indicated that, in the absence of piscine predation, intraspecific competition might be an important selective factor in ponds (Gonda, Herczeg & Merilä, 2009; Herczeg, Gonda & Merilä, 2009a, b; Herczeg *et al.*, 2012). However, it is not currently known whether nine-spined sticklebacks express phenotypic plasticity in defence traits and, if so, whether the extent of plasticity varies among populations from different habitats.

The goal of the present study was to investigate variation in phenotypic plasticity of morphological traits of predator-adapted marine vs. competition-adapted pond nine-spined stickleback populations reared under different levels of perceived predation risk and food supply in a factorial common garden experiment. More specifically, we aimed to disentangle the effects of population history, rearing environment and sex on body shape and body armour variation. We hypothesized that (1) fish from marine populations – locally adapted to the high and potentially variable piscine predation – would show stronger predator-induced plasticity than fish from pond populations that are locally adapted to a stable, piscine predator free environment. We also hypothesized that (2) if predator-induced plasticity occurs and is limited by resource availability, it should be more strongly expressed under good rather than poor feeding conditions.

MATERIAL AND METHODS

Sampling sites

Adult nine-spined stickleback were collected from two pond and four marine sites (Fig. 1) during

*Corresponding author. E-mail: kaisa.valimaki@helsinki.fi
†Present address: Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/C, H-1117, Hungary.

© 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **107**, 854–866

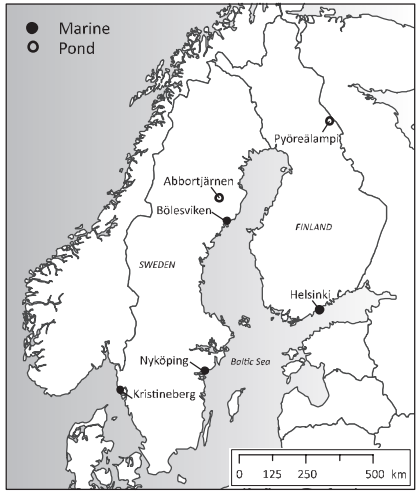


Figure 1. Map showing the nine-spined stickleback populations used in the study. Open circles represent pond populations, marine populations are marked with filled circles.

the breeding season in 2009 using minnow traps and seine nets. Marine sampling sites, excluding Bölesviken, were shallow coastal bays. Bölesviken fish were collected from a creek that was directly connected to the sea. Marine stickleback are sympatric with a large number of predatory and competitor fish species. The pond sampling sites (Pyöreälampi and Abbortjärnen) were small (surface area < 5 ha) ponds that are free of any sympatric fish species. A small number of recently introduced whitefish (*Coregonus lavaretus*) may occur in Pyöreälampi in low density, but, as whitefish are mostly planktivorous, we expect them to be competitors rather than predators to the nine-spined stickleback (Kahilainen *et al.*, 2004). The ponds are structurally very simple habitats that contain little vegetation and few fallen logs and rocks on the bottom. Although we did not quantify stickleback numbers in the different habitats, stickleback densities in the ponds are much higher than those in marine sites. Because of their small size, ponds do not sustain permanent populations of any piscivorous bird species, although they sporadically might visit the ponds for feeding. In all sampled sites, predation by aquatic insects and cannibalism is likely to be present.

Common garden experiment

Adult fish were transported to the fish-breeding facilities of the University of Helsinki and kept at +17 °C under constant light regime. Fish were fed frozen bloodworms (Chironomid larvae) in excess until they reached reproductive condition. Six to ten artificial crosses per population were performed between 1 July and 3 August 2009, (number of families: Abbortjärnen, 6; Bölesviken, 6; Helsinki, 8; Kristineberg, 10; Nyköping, 8; Pyöreälampi, 7). The crosses were performed by over anaesthetizing the males in tricaine methanesulphonate (MS222) and dissecting out their testicles. Testicles were minced in a drop of water and the solution was pipetted on top of the egg clumps that were gently pushed out from ripe females. The clutches were divided into two, and half of the replicates were placed in containers with water containing olfactory cues from a predator fish (see below) and half of them into containers with clean water in four Allentown Zebrafish Rack Systems (Aquaneering Inc., San Diego, CA, USA, hereafter 'rack'). After the fish reached their free-swimming stage – when they also start feeding – they were individually allocated into random 1.4-L containers in the racks. One hundred containers were used in every rack, totalling up to 400 containers. Four treatment combinations (see below) were applied and we aimed to have equal population/family representation in each of them. Mortality occurred mainly at the early life stages and, after 240 days of rearing, 304 individuals were available for the analyses. The photoperiod was set to 14 : 10 h (light : dark) and the water temperature was held constant at 12 °C. Stickleback were initially fed with living brine shrimp (*Artemia salina*) nauplii and, after 80 days, gradually changed to frozen bloodworms. All rearing took place in freshwater as the salinity of the Baltic marine sites is very low (*c.* 0–6.0 *psu*; Shimada *et al.*, 2011) and nine-spined stickleback routinely breed in creeks and at creek inlets.

Treatments

Fish were divided randomly and evenly into four treatments in a factorial experiment with two predation and two food level treatments. For the predation treatment, all racks were fitted with an external 150-L plastic tank. Water (after being filtered by biological and mechanical filters) was pumped through these extra tanks before it returned to the rack and was pumped to the individual containers. Two 10- to 15-cm long perches (*Perca fluviatilis*) were placed into two randomly chosen tanks to provide the olfactory stimulus to the test fish (predator present treatment). Two remaining extra tanks served as controls without predator (predator absent treatment). Perches were fed with frozen bloodworms. Note that perch is an abundant predator in the coastal areas of Baltic Sea (Ådjers *et al.*, 2006), and perhaps the most abundant piscine predator of the Fennoscandian freshwaters (Koli, 1990). For the food treatment, fish within families, predation treatments and racks were divided randomly into high (fed twice a day in excess) and low (fed once in 2 days in excess) food treatment groups. As stickleback do not eat the bloodworms after a few hours, food remaining in the tank after feeding is a sign that the fish have been fed to excess.

Measurements

Fish were over-anaesthetized with MS 222 at the age of 34 weeks and photographed immediately. All photographs were taken with a digital camera on a tripod under standardized conditions. Landmarks (see below, Fig. 2) were marked with insect pins and a piece of millimetre paper was placed in every photograph for scaling. Nine-spined stickleback in our study populations possess distinct anterior and posterior lateral plates. The number of posterior plates was counted under a dissecting microscope. Length of pelvic spines was measured three times from both sides with a digital calliper (pelvic girdles were measured from the photographs, see below). Sex was verified from the gonads via dissection.

Landmark based geometric morphometrics (Bookstein, 1991; Rohlf, 1999; Zelditch *et al.*, 2004) were used to analyse shape variation. Landmarks were chosen so that they would capture most of the shape variation with a minimum set of variables to maximize the power of the geometric morphometric analyses. We used software tpsDig 2.15 (Rohlf, 2006) to digitize landmarks from the images. The 12 landmarks used are listed and depicted in Figure 2. In addition, an extra landmark (x) was placed at the caudal tip of the posterior process of the pelvic girdle on the ventral midline (see Fig. 2) to measure the length of the pelvic girdle. Pelvic girdle length and the standard length of the fish (from the end of the tail base to the tip of the snout) were measured from the digital photographs with the aid of tpsDig 2.15.

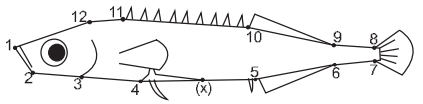


Figure 2. Illustration of the landmark positions for geometric morphometric measurements: 1, anterior tip of upper lip; 2, posterior edge of angular process; 3, anterior tip of ectocoracoid; 4, posterior tip of ectocoracoid; 5, base of the first anal ray on ventral midline (VML); 6, insertion of anal fin membrane on VML; 7, origin of caudal fin membrane on VML; 8, origin of caudal fin membrane on dorsal midline (DML); 9, insertion of dorsal fin membrane on DML; 10, base of the first dorsal fin ray on DML; 11, anterior junction of the first dorsal spine on DML; 12, posterior extent of the supraoccipital; x, a separate landmark that was used to measure pelvic girdle length but was not used in shape analysis.

Statistical analysis

We used TpsRelw 1.46 (Rohlf, 2006) to superimpose the digitized landmarks. TpsRelw applies a generalized orthogonal least-squares Procrustes procedure to align, scale and rotate the landmark configurations (Rohlf & Slice, 1990). Both partial warp scores and relative warp (RW) scores were obtained. Relative warp analysis with equal weight at all spatial scales ($\alpha = 0$) is basically a principal component analysis (PCA) performed on all partial warps and uniform components. TpsRelw was used to calculate centroid size, which is the square root of the sum of the squared distances from the centroid to each landmark (Bookstein, 1991) and widely used as a reliable measure of overall body size (e.g. Walker, 1997; Langerhans & DeWitt, 2004; Leinonen *et al.*, 2006). Centroid size and standard length showed strong and positive correlation in our data ($r = 0.98$, $P < 0.001$, $n = 303$), and thus we used centroid size as the body size variable in our analyses.

To analyse general patterns of shape differentiation, we first ran a multivariate general linear model (GLM) on all partial warps and uniform components, with population, sex and treatments as fixed factors and centroid size as a covariate. All interactions between factors up to three-way level were included in the model. Second, we ran separate general linear mixed models (GLMMs) on the relative warps that captured more than 10% of the total variation (e.g. Berner, Grandchamp & Hendry, 2009), with habitat, sex and treatments as fixed factors and centroid size as a covariate. Again, we included all two- and three-way interactions between factors. We also added population nested within habitat as a random factor to control for the non-independence of populations within habitat type and family nested within population as a random factor to control for the non-independence of individuals within families. We started with the full models and used backward stepwise model selection based on the $P < 0.05$ criterion. This method is considered as a conservative one among the several options for model selection (Murtaugh, 2009). This was carried out by first removing non-significant three-way interactions in order of decreasing *P*-value and then we moved to two-way

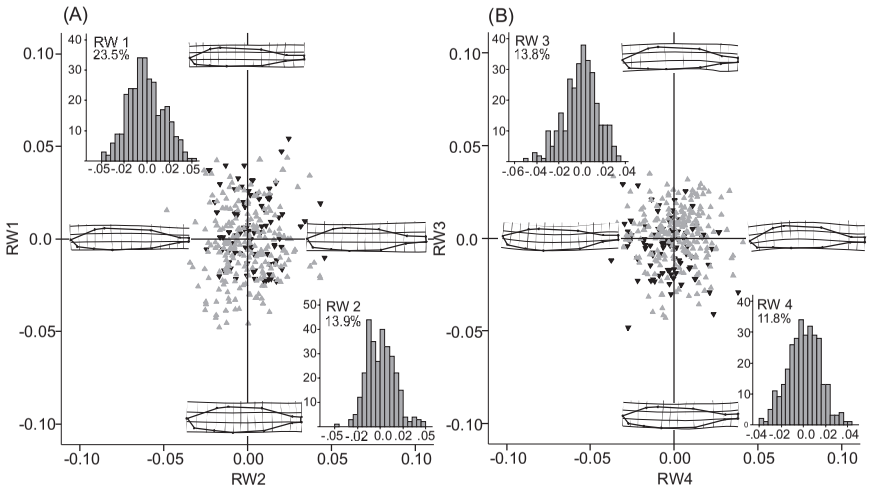


Figure 3. Results from the geometric morphometric analyses. The four relative warps (RWs) that describe more than 10% of the total variation are shown. The trends are visualized by plotting the values of (A) RW1 against RW2 and (B) RW3 against RW4. Grey triangles represent marine populations, black triangles are pond populations. Drawings of the extreme body shapes along each relative warp and histograms show the distributions of the relative warp scores.

interactions and finally to main effects. We did not remove any two-way interactions that were included in significant three-way interactions or main effects involved in any significant interactions.

We used size-corrected estimates of length of pelvic girdle and spine (none of the fish in our samples lacked spines or girdles) by calculating standardized residuals from their linear regressions on centroid size. Residual pelvic girdle and spine length were then analysed, together with number of lateral plates in a PCA to obtain a smaller set of fully independent variables. The obtained principal component scores were then analysed using GLMMs with habitat, sex and treatments as fixed factors and population nested within habitat and family nested within population as random factors. All interactions up to three-way level were analysed by applying the model selection procedures explained above. All statistical analyses were performed using PASW 18 (SPSS Inc., Chicago, IL, USA).

RESULTS

Body shape

The multivariate GLM revealed that there was overall differentiation in shape between populations and sexes (population: Wilk's $\lambda = 0.04$, $P < 0.001$; sex: Wilk's $\lambda = 0.51$, $P < 0.001$; population × sex: Wilk's $\lambda = 0.52$, $P < 0.001$). Also, centroid size was significantly different (Wilk's $\lambda = 0.66$, $P < 0.001$). None of the other effects were significant (all $P > 0.05$, results not shown).

The first four RWs described approximately 63% of the total variance in shape (Fig. 3). The first RW explained 23.54% of the variance and described a gradient from fish with small heads, shallow bodies and long caudal peduncles towards fish with large heads, deep bodies and short caudal peduncles (Fig. 3). RW2 explained 13.94% of the variance and described a gradient from fish with elongated mid-bodies and short caudal peduncles towards fish with short mid-bodies and long caudal peduncles (Fig. 3). RW3 explained 13.38% of the variance and described variation in the length of dorsal and anal fins (Fig. 3). RW4 explained 11.8% of the variance and captured variation related to bending.

GLMM of the first RW revealed no significant effect of predator treatment (Table 1). However, the effects of habitat (Fig. 4a, Table 1), sex (Fig. 4b, Table 1) food treatment (Fig. 4c, Table 1) and centroid size (Table 1) were all significant. Both males and fish originating from pond populations had larger heads, deeper bodies and shorter caudal peduncles than females or marine individuals, respectively. Fish from the high food treatment also tended to have relatively larger heads, deeper bodies and shorter caudal peduncles than fish from low food treatment, but this effect was much weaker than what is seen between habitats or sexes (Fig. 4). None of the other fixed effects were significant (Table 1). Population effect was also non-significant ($Z = 0.72$, $P = 0.47$), whereas there was a significant family effect ($Z = 2.83$, $P = 0.005$). There was no significant effect for the second RW apart from that of the centroid size (Table S1 and Fig. S1). Sex and centroid size had a significant effect on the third RW, while the other effects were non-significant (Table S1 and Fig. S1). In RW3, females seemed to have shorter anal and dorsal fins and also slightly higher stem of the caudal peduncle than males, while the other effects were non-significant (see also Supporting Information, Table S1 and Fig. S1). The fourth RW described mainly bending, and there was a significant sex effect showing slight upwards bend in females, whereas males were bent downwards (see also Supporting Information: Table S1 and Fig. S1).

Table 1. Results of the general linear mixed model of nine-spined stickleback body shape variation as captured by the first relative warp (RW1, see Fig. 3). Backward stepwise model selection was applied. Non-significant effects (when not being part of a higher level significant interaction) are shown from the one-by-one back-substitution to the final model

Effect	Numerator d.f.	Denominator d.f.	*F*	*P*
Habitat	1	5.8	27.885	0.002
Sex	1	276.1	87.837	< 0.001
Predator	1	277.6	0.005	0.946
Food	1	268.9	5.268	0.022
Habitat × predator	1	275.7	0.269	0.604
Habitat × food	1	262.4	1.815	0.179
Habitat × sex	1	270.6	2.425	0.121
Predator × food	1	259.1	0.449	0.503
Predator × sex	1	273.8	0.102	0.750
Feeding × sex	1	268.7	3.049	0.082
Habitat × predator × food	1	257.1	0.053	0.818
Habitat × predator × sex	1	281.1	0.006	0.937
Habitat × feeding × sex	1	271.5	1.182	0.278
Predator × feeding × sex	1	271.2	0.145	0.704
Centroid size	1	164.6	70.043	< 0.001

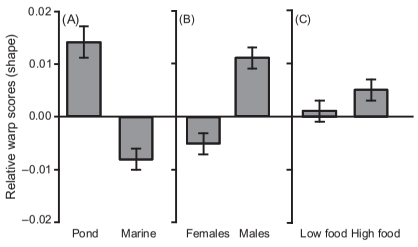


Figure 4. Least squares means (±SE) for the significant terms in the general linear mixed model of the first relative warp describing body shape variation in nine-spined stickleback (for a visual interpretation of the first relative warp, see Fig. 3). High scores represent stickleback with big heads, deep bodies and short caudal peduncles.

Armour traits

The PCA run of the three armour variables retrieved one principal component with eigenvalue > 1, describing 60% of the total variation (factor loadings: number of lateral plates = 0.63, relative pelvic spine length = 0.85, relative pelvic girdle length = 0.83, eigenvalue = 1.80). This first principal component captured variability in the amount of bony armour, describing a gradient of individuals with relatively small pelvic girdles, short pelvic spines and low number of lateral plates towards individuals with large girdles, long spines and high number of plates. The GLMM of the principal component revealed no effect of predator treatment, but there was a significant habitat × sex interaction, revealing that (1) pond fish were less armoured than marine fish and (2) females were more armoured than males in the

marine but not in the pond habitat (Fig. 5, Table 2). All other fixed effects in the model were non-significant (Table 2; Fig. S1). Random family effect was significant ($Z = 2.86$, $P = 0.004$), while the population effect was not ($Z = 1.33$, $P = 0.18$).

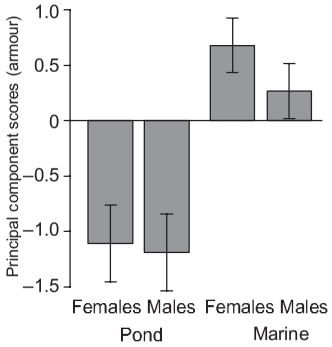


Figure 5. Least squares means (±SE) for the significant habitat × sex interaction in the general linear mixed model of the first principal component describing the variation in bony armour traits (pelvic spine length, pelvic girdle length and lateral plate number) in nine-spined stickleback. High scores imply long pelvic spines and girdles and more lateral plates (see Table 2).

DISCUSSION

The main aim of this study was to explore the possible existence of predator-induced plasticity in body shape and armour in nine-spined stickleback originating from populations differing in predation risk, as well as the possible influence of energy constraints on expression of this plasticity. We expected that populations adapted to predation would show a higher degree of plasticity towards variation in perceived predatory risk than populations not adapted to predation. This is because spatial or temporal environmental variation is likely to maintain phenotypic plasticity (Moran, 1992), whereas lack of variation is suggested to decrease it, possibly through genetic assimilation (Waddington, 1953a; Crispo, 2007; Aubret & Shine, 2009; Pfennig *et al.*, 2010). We also expected that any predation-induced plasticity would be constrained by low food treatment. In contrast to our expectations, we could not detect any predation-induced plastic responses to our treatments. However, we found strong morphological divergence between the habitat types and sexes, in line with earlier research on the same study system (Herczeg *et al.*, 2010; M. Turtiainen, G. Herczeg & J. Merilä, unpubl. data). Marine fish had relatively smaller heads and more streamlined bodies, with longer caudal peduncles, more lateral plates, and relatively larger pelvic girdles and longer pelvic spines than pond fish. Females had relatively smaller heads, were more streamlined and had longer caudal peduncles, shorter dorsal and anal fins and stronger upward bend than males. We also found a habitat-dependent sex effect

Table 2. Results of the general linear mixed model of the first principal component (PC1) of nine-spined stickleback armour traits. PC1 describes a gradient from fish with reduced armour towards fish with full armour (see Table 2). Backward stepwise model selection was applied. Non-significant effects (when not being part of a higher level significant interaction) are shown from the one-by-one back-substitution to the final model

Effect	Numerator d.f.	Denominator d.f.	F	P
Habitat	1	4.1	12.725	0.023
Sex	1	276	12.76	< 0.001
Predator	1	275.1	2.101	0.148
Food	1	268	3.036	0.083
Habitat × predator	1	280.6	1.682	0.196
Habitat × food	1	266.6	0.1	0.752
Habitat × sex	1	276	8.072	0.005
Predator × food	1	264.3	2.561	0.111
Predator × sex	1	277.5	1.092	0.297
Feeding × sex	1	273.7	0.882	0.349
Habitat × predator × food	1	260.2	0.007	0.935
Habitat × predator × sex	1	283.7	0.273	0.602
Habitat × feeding × sex	1	276.8	1.069	0.302
Predator × feeding × sex	1	274.8	0.385	0.535

in the body armour: marine females were more armoured than marine males, while there was no sexual dimorphism in armouring in pond fish. In general, these results conform to a pattern where evolutionary flexibility (namely, divergence between and within populations) is coupled with a high degree of developmental rigidity (namely, lack of environment-induced plasticity) in the studied traits.

One explanation for not being able to detect any predator-induced plasticity in nine-spined stickleback morphology could be that the experimental fish failed to identify the smell of perch as a threat of predation. Plastic responses to predator presence are often induced by chemical cues, either the smell of predator per se, and/or the alarm cues of the conspecifics (Wisenden & Chivers, 2006). The smell of a predator alone might not be enough to induce plastic response in the prey, but alarm cues are needed as well (Brönmark & Pettersson, 1994). However, the smell of predator (perch) without alarm cues is enough to induce plasticity in body shape and growth in the three-spined stickleback (Frommen *et al.*, 2011). Here, the smell of live perch was also used as an olfactory cue without alarm cues, but no response in morphology was detected. However, the nine-spined stickleback from this same experiment have shown predator-induced plasticity in other traits. In the presence of the olfactory cues from perch, stickleback became less aggressive and risk taking, and their growth decreased (Herczeg & Välimäki, 2011; Välimäki & Herczeg, 2012), supporting that they can identify perch as a possible threat. Therefore, it appears that nine-spined stickleback, originating either from populations sympatric to piscine predators or populations with no piscine predation, do not express predator-induced plasticity in body shape or armour.

The extensive research carried out on armour variation of three-spined stickleback suggests that populations living in sympatry with predators benefit from robust armouring (e.g. Hagen & Gilbertson, 1972; Moodie, 1972; Gross, 1978), as it offers effective protection against gape limited predation (Hoogland *et al.*, 1957; Reimchen, 1991; Lescak & von Hippel, 2011). Compared with the three-spined stickleback, the role of armour as a protection against predators in nine-spined stickleback is less important as both spines and lateral plates are smaller in size (Hoogland *et al.*, 1957). If the role of armour as a means of protection from predators is relatively small, the costs of plasticity (maintaining the ability to express phenotypic plasticity and actually expressing it) might outweigh the benefits (DeWitt *et al.*, 1998). In such situations, stabilizing selection can lead to phenotypes that are fixed irrespective of the environment (i.e. canalization, Waddington, 1953a; Crispo, 2007). However, experiments have shown that nine-spined stickleback armour is still a significant anti-predator defence (Hoogland *et al.*, 1957; Ziuganov & Zotin, 1995). Further, the somewhat limited anti-predatory use of body armour in nine-spined stickleback does not mean that it is unrelated to predation pressure in the wild. Previous work has established that there is large habitat-dependent variation in nine-spined stickleback armour (McPhail, 1963; Gross, 1979; Herczeg *et al.*, 2010; Mobley *et al.*, 2011): fish from large lakes and marine environments facing a number of sympatric predatory fish have fully developed pelvic apparatuses with long girdle and spines, as well as a long row of posterior lateral plates. In contrast, their pond conspecifics lacking sympatric piscine predators often have reduced pelvic apparatuses (pelvic girdle and spines are shorter, or even completely absent) and a lower number of posterior lateral plates (Herczeg *et al.*, 2010). Besides phenotypic variation, the genetic basis of armour reduction following the migration from ancestral marine habitats to freshwater environments has been uncovered in the three-spine stickleback (Cresko *et al.*, 2004; Colosimo *et al.*, 2005; Shapiro, Bell & Kingsley, 2006; Chan *et al.*, 2010). Following this, genomic areas involved in the armour reduction in nine-spined stickleback have been also identified, but the patterns are not identical to those of three-spined stickleback (Shapiro *et al.*, 2009). The present study reinforces the importance of genetic variation behind phenotypic patterns in nine-spined stickleback morphology by demonstrating that it is not plastic under different environmental conditions, while suggesting a genetic basis of the habitat and sex divergence. The significant family effects within population further suggest the evolvability of body armour in nine-spine stickleback; however, based on our simple design using F1 full-sib families, maternal effects cannot be excluded either.

Energy supply can play a role in the expression of phenotypic plasticity, as the energetic state of an individual can impact development of morphological traits (Borcherding & Magnhagen, 2008) and type of food has been shown to cause variable plastic responses in morphological characters (Day & McPhail, 1996; Andersson, Johansson & Söderlund, 2006; Parsons & Robinson, 2007; Berner *et al.*, 2008; Wund *et al.*, 2008, 2012). In the present experiment, the food treatments represented a fourfold difference in food availability, resulting in marked differences in growth and behaviour (Herczeg & Välimäki, 2011; Välimäki & Herczeg, 2012). Growth of the fish was significantly faster in the high compared with the low food treatment. There was also a sex × food treatment interaction: females responded to the high food treat-

ment more strongly than males. Response was also dependent on the predation risk treatment: fish utilized the larger food supply only in the absence of predator cues (Välimäki & Herczeg, 2012). It was also shown that food restriction increased feeding activity and made pond fish become more risk taking (Herczeg & Välimäki, 2011). Still, the present study uncovered only a weak food treatment effect on body shape and nothing on the armour traits. While it has been suggested that pond and marine populations used here differ in the level of intraspecific competition (Gonda *et al*., 2009; Herczeg *et al*., 2009a, b, 2012; Välimäki & Herczeg, 2012), and thus might have adapted to different levels of resource competition, we did not observe any habitat specific food treatment effects in line with this expectation.

Body shape is known to differ between marine/lake vs. pond nine-spined stickleback (Herczeg *et al*., 2010; Mobley *et al*., 2011). This variation, i.e. elongated body with long caudal peduncles in the marine/lake vs. deep body with short caudal peduncle in ponds, agrees with the results of the present study. These patterns correspond to the body shape variation reported in three-spined stickleback (Reimchen, Stinson & Nelson, 1985; Bell & Foster, 1994; Walker & Bell, 2000; Hermida *et al*., 2005; Kristjánsson, 2005; Leinonen *et al*., 2006; Aguirre *et al*., 2008; Sharpe *et al*., 2008; Berner *et al*., 2009), following predictions drawn from the relationship between optimal swimming performance and habitat utilization. In pelagic/limnetic habitats without refugia, fish body shape is optimized for prolonged steady swimming. In benthic habitats, which usually have numerous refugia, fish body shape is adapted for increased manoeuvrability and short but fast bursts of swimming to reach the refugia quickly (e.g. Webb, 1983; Walker, 1997; Bergstrom, 2002; Walker *et al*., 2005). Further, there are numerous examples for predation-related phenotypic plasticity in fish body shape from three-spined stickleback (Frommen *et al*., 2011) and other species (Brönmark & Miner, 1992; Chivers *et al*., 2007; Abate, Eng & Kaufman, 2010). In this light, and considering the treatment effects on body size (Välimäki & Herczeg, 2012), the lack of body shape plasticity found here was unexpected.

Besides the habitat-dependent population divergence in armour traits, strong sexual dimorphism in body shape and habitat-specific sexual dimorphism in armour traits were found. For the body shape, females were characterized by smaller heads, more streamlined bodies, shorter dorsal and anal fins, longer caudal peduncles and stronger bending than males. The sex-specific differences in armour traits (females having longer spines, more plates and larger pelvic girdle than males) were only visible in marine populations. These patterns follow by and large the previous results from other stickleback species (Caldecutt & Adams, 1998; Leinonen *et al*., 2006; Kitano, Mori & Peichel, 2007; Aguirre & Akinpelu, 2010; Leinonen, Cano & Merilä, 2011a) and previously observed patterns from wild nine-spined stickleback (M. Turtiainen, G. Herczeg & J. Merilä, unpubl. data). The sex-specific variation in armour and body shape is thought to derive from the differences in life histories and behavioural roles. In the three-spined stickleback, males are more benthic and females more limnetic in their lifestyle (Spoljaric & Reimchen, 2008). Hence, the body shape divergence between sexes can represent adaptations to the different habitats (e.g. Walker, 1997; Leinonen *et al*., 2011a). The lack of sex-specific differences in armour traits of pond fish most likely reflects the absence of fish predation. Once the benefits from having armour are smaller, there is no directional selection that would sustain the divergence between sexes. The close resemblance in body shape divergence between marine and pond fish compared with differences between males and females might also be explained by the genetic correlations between certain components of body shape. Genetic correlations can constrain the direction of shape evolution, forcing similar shifts in traits, even when the evolutionary forces affecting them are different (Lande, 1979; Arnold, 1992). This hypothesis has been evaluated using three-spined stickleback where ancestral (marine) genetic variation could steer the armour and body shape evolution in derived populations, but the results have been mixed (Schluter, 1996; Berner *et al*., 2010; Leinonen, Cano & Merilä, 2011b; Aguirre & Bell, 2012). The reason for the observed sex-specific body curvature remains unsolved.

In summary, we found that perceived predation risk from a common piscine predator did not lead to induced expression of morphological defences in the nine-spined stickleback, irrespective of whether the experimental fish originated from populations exposed or unexposed to piscine predation. Likewise, food availability did not influence the expression of plasticity in armour traits and had only a minor effect on body shape. The lack of predator-induced plasticity is somewhat unexpected, given that (1) predation is well known for inducing morphological defences in a wide variety of taxa (e.g. Stemberger & Gilbert, 1984; Brönmark & Miner, 1992; Tollrian, 1995; Van Buskirk & Schmidt, 2000; Hill & Hill, 2002; Young, Stanton & Christian, 2003) and (2) the studied traits showed marked treatment-independent variation both among (habitat-dependent divergence) and within (sexual dimorphism) populations. It seems that nine-spine stickleback morphology is evolvable, but ontogenetically resistant in the face of variation in predation risk.

ACKNOWLEDGEMENTS

Thanks are due to Göran Englund, Jacquelin DeFaveri, Jenni Hämäläinen, Lotta Kvarnemo, Pia Rännänen and Pirkko Siikamäki, who helped to obtain the parental fish. Nurul Izza Ab Ghani, Maarit Haataja, Marika Lilja, Linda Uoti and, especially, Mirva Turtiainen are thanked for their invaluable help with fish maintenance. The manuscript was greatly improved by comments from Tuomas Leinonen and three anonymous reviewers. Our work was supported by the Academy of Finland (G.H., no. 128716; J.M., #134728 and #129662), the Finnish Graduate School in Population Genetics, Ella and Georg Ehrnrooth Foundation and Eemil Aaltonen foundation (K.V.). The research leading to these results has also benefited from funding by the European Community's Seventh Framework Programme (FP/2007–2013) under grant agreement no. 217246 made with BONUS, the joint Baltic Sea research and development programme (J.M.). The experiment was conducted under the license of the Finnish National Animal Experiment Board (licence no. STH223A).

REFERENCES

Abate ME, Eng AG, Kaufman L. 2010. Alarm cue induces an anti-predator morphological defense in juvenile Nicaragua cichlids *Hypsophrys nicaraguensis*. *Current Zoology* **56:** 36–42.

Ådjers K, Appelberg M, Eschbaum R, Lappalainen A, Minde A, Repecka R, Thoresson G. 2006. Trends in coastal fish stocks of the Baltic Sea. *Boreal Environment Research* **11:** 13–25.

Aguirre WE, Akinpelu O. 2010. Sexual dimorphism of head morphology in three-spined stickleback *Gasterosteus aculeatus*. *Journal of Fish Biology* **4:** 802–821.

Aguirre WE, Bell M. 2012. Twenty years of body shape evolution in a three-spine stickleback population adapting to a lake environment. *Biological Journal of the Linnean Society* **105:** 817–831.

Aguirre WE, Ellis KE, Kusenda M, Bell MA. 2008. Phenotypic variation and sexual dimorphism in anadromous three-spine stickleback: implications for postglacial adaptive radiation. *Biological Journal of the Linnean Society* **95:** 465–478.

Andersson J, Johansson F, Söderlund T. 2006. Interactions between predator- and diet-induced phenotypic changes in body shape of crucian carp. *Proceedings of The Royal Society B* **273:** 431–437.

Arnold SJ. 1992. Constraints on phenotypic evolution. *American Naturalist* **140:** S85–S107.

Aubret F, Shine R. 2009. Genetic assimilation and the postcolonization erosion of phenotypic plasticity in island tiger snakes. *Current Biology* **19:** 1932–1936.

Bell MA, Foster SA, eds. 1994. *The evolutionary biology of the three-spine stickleback*. New York: Oxford University press.

Bergstrom CA. 2002. Fast-start swimming performance and reduction in lateral plate number in three-spine stickleback. *Canadian Journal of Zoology* **80:** 207–213.

Berner D, Adams DC, Grandchamp A-C, Hendry AP. 2008. Natural selection drives patterns of lake-stream divergence in stickleback foraging morphology. *Journal of Evolutionary Biology* **21:** 1653–1665.

Berner D, Grandchamp A-C, Hendry AP. 2009. Variable progress toward ecological speciation in parapatry: stickleback across eight lake–stream transitions. *Evolution* **63:** 1740–1753.

Berner D, Roesti M, Hendry AP, Salzburger W. 2010. Constraints on speciation suggested by comparing lake–stream stickleback divergence across two continents. *Molecular Ecology* **19:** 4963–4978.

Bookstein FL. 1991. *Morphometric tools for landmark data: geometry and biology*. Cambridge: Cambridge University Press.

Borcherding J, Magnhagen C. 2008. Food abundance affects both morphology and behaviour of juvenile perch. *Ecology of Freshwater Fish* **17:** 207–218.

Brönmark C, Miner JG. 1992. Predator-induced phenotypical change in body morphology in crucian carp. *Science* **258:** 1348–1350.

Brönmark C, Pettersson LB. 1994. Chemical cues from piscivores induce a change in morphology in crucian carps. *Oikos* **70:** 396–402.

Caldecutt WJ, Adams DC. 1998. Morphometrics of trophic osteology in the three-spine stickleback *Gasterosteus aculeatus*. *Copeia* **4:** 827–838.

Chan YF, Marks ME, Jones FC, Villarreal G, Shapiro MD, Brady SD, Southwick AM, Absher DM, Grimwood J, Schmutz J, Myers RM, Petrov D, Jónsson B, Schluter D, Bell MA, Kingsley DM. 2010. Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a Pitx1 enhancer. *Science* **327:** 302–305.

Chivers DP, Zhao X, Brown GE, Marchant TA, Ferrari MCO. 2007. Predator-induced changes in morphology of a prey fish: the effects of food level and temporal frequency of predation risk. *Evolutionary Ecology* **22:** 561–574.

Colosimo PF, Hosemann KE, Balabhadra S, Villarreal G, Dickson M, Grimwood J, Schmutz J, Myers RM, Schluter D, Kingsley DM. 2005. Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. *Science* **307:** 1928–1933.

Cresko WA, Amores A, Wilson C, Murphy J, Currey M, Phillips P, Bell MA, Kimmel CB, Postlethwait JH. 2004. Parallel genetic basis for repeated evolution of armor loss in Alaskan three-spine stickleback populations. *Proceedings of the National Academy of Sciences of the United States of America* **101:** 6050–6055.

Crispo E. 2007. The Baldwin effect and genetic assimilation: revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. *Evolution* **61:** 2469–2479.

Day T, Mcphail JD. 1996. The effect of behavioural and morphological plasticity on foraging efficiency in the

three-spine stickleback (*Gasterosteus* sp). *Oecologia* **108:** 380–388.

DeWitt TJ, Sih A, Wilson DS. 1998. Costs and limits of phenotypic plasticity. *Trends in Ecology and Evolution* **13:** 77–81.

Domenici P, Turesson H, Brodersen J, Brönmark C. 2008. Predator-induced morphology enhances escape locomotion in crucian carp. *Proceedings of the Royal Society B* **275:** 195–201.

Frommen JG, Herder F, Engqvist L, Mehlis M, Bakker TCM, Schwarzer J, Thünken T. 2011. Costly plastic morphological responses to predator specific odour cues in three-spined sticklebacks (*Gasterosteus aculeatus*). *Evolutionary Ecology* **25:** 641–656.

Gonda A, Herczeg G, Merilä J. 2009. Habitat-dependent and -independent plastic responses to social environment in the nine-spine stickleback (*Pungitius pungitius*) brain. *Proceedings of the Royal Society B* **276:** 2085–2092.

Gross HP. 1978. Natural selection by predators on the defensive apparatus of the three-spined stickleback, *Gasterosteus aculeatus L. Canadian Journal of Zoology* **56:** 398–413.

Gross HP. 1979. Geographic variation in European nine-spine sticklebacks, *Pungitius pungitius*. *Copeia* **1979:** 405–412.

Hagen DW, Gilbertson LG. 1972. Geographic variation and environmental selection in *G. aculeatus* L. in the Pacific Northwest America. *Evolution* **26:** 32–51.

Harvell CD. 1990. The ecology and evolution of inducible defences. *The Quarterly Review of Biology* **65:** 323–340.

Herczeg G, Välimäki K. 2011. Intraspecific variation in behaviour: effects of evolutionary history, ontogenetic experience and sex. *Journal of Evolutionary Biology* **24:** 2434–2444.

Herczeg G, Gonda A, Merilä J. 2009a. Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *Journal of Evolutionary Biology* **22:** 544–552.

Herczeg G, Gonda A, Merilä J. 2009b. The social cost of shoaling covaries with predation risk in nine-spined stickleback, *Pungitius pungitius*, populations. *Animal Behaviour* **77:** 575–580.

Herczeg G, Turtiainen M, Merilä J. 2010. Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. *Biological Journal of Linnean Society* **101:** 403–416.

Herczeg G, Gonda A, Kuparinen A, Merilä J. 2012. Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? *Evolutionary Ecology* **26:** 109–122.

Hermida M, Fernández C, Amaro R, San Miguel E. 2005. Morphometric and meristic variation in Galician three-spine stickleback populations, north-west Spain. *Environmental Biology of Fishes* **73:** 189–200.

Hill MS, Hill AL. 2002. Morphological plasticity in the tropical sponge *Anthosigmella varians*: responses to predators and wave energy. *Biological Bulletin* **202:** 86–95.

Hoogland R, Morris D, Tinbergen N. 1957. The spines of sticklebacks (*Gasterosteus* and *Pygosteus*) as means of defence against predators (*Perca* and *Esox*). *Behaviour* **10:** 205–236.

Januszkiewicz AJ, Robinson BW. 2007. Divergent walleye (*Sander vitreus*) – mediated inducible defenses in the centrarchid pumpkinseed sunfish (*Lepomis gibbosus*). *Biological Journal of Linnean Society* **90:** 25–36.

Johansson F. 2002. Reaction norms and production costs of predator-induced morphological defences in a larval dragonfly (*Leucorrhinia dubia*: *Odonata*). *Canadian Journal of Zoology* **80:** 944–950.

Kahilainen K, Malinen T, Tuomaala A, Lehtonen H. 2004. Diel and seasonal habitat and food segregation of three sympatric *Coregonus lavaretus* forms in a subarctic lake. *Journal of Fish Biology* **64:** 418–434.

Kawecki TJ, Ebert D. 2004. Conceptual issues in local adaptation. *Ecology Letters* **7:** 1225–1241.

Kishida O, Trussell GC, Nishimura K. 2007. Geographic variation in a predator-induced defense and its genetic basis. *Ecology* **88:** 1948–1954.

Kitano J, Mori S, Peichel CL. 2007. Sexual dimorphism in the external morphology of the three-spine stickleback (*Gasterosteus aculeatus*). *Copeia* **2007:** 336–349.

Koli L. 1990. *Suomen kalat*. Helsinki: Werner Söderström.

Kristjánsson BK. 2005. Rapid morphological changes in three-spine stickleback, *Gasterosteus aculeatus*, in fresh water. *Environmental Biology of Fishes* **74:** 357–363.

Laforsch C. 2004. Inducible defences in multipredator environments: cyclomorphosis in *Daphnia cucullata*. *Ecology* **85:** 2302–2311.

Lahti DC, Johnson NA, Ajie BC, Otto SP, Hendry AP, Blumstein DT, Coss RG, Donohue K, Foster SA. 2009. Relaxed selection in the wild. *Trends in Ecology and Evolution* **24:** 487–496.

Lande R. 1979. Quantitative genetic analysis of multivariate evolution, applied to brain–body size allometry. *Evolution* **33:** 402–416.

Langerhans RB, DeWitt TJ. 2004. Shared and unique features of evolutionary diversification. *American Naturalist* **164:** 335–349.

Laurila A, Pakkasmaa S, Merilä J. 2006. Population divergence in growth rate and anti-predator defences in *Rana arvalis*. *Oecologia* **147:** 585–595.

Leinonen T, Cano JM, Mäkinen H, Merilä J. 2006. Contrasting patterns of body shape and neutral genetic divergence in marine and lake populations of threespine sticklebacks. *Journal of Evolutionary Biology* **19:** 1803–1812.

Leinonen T, Cano JM, Merilä J. 2011a. Genetic basis of sexual dimorphism in the three-spine stickleback *Gasterosteus aculeatus*. *Heredity* **24:** 218–227.

Leinonen T, Cano JM, Merilä J. 2011b. Genetics of body shape and armour variation in three-spine sticklebacks. *Journal of Evolutionary Biology* **24:** 206–218.

Lescak EA, von Hippel FA. 2011. Selective predation of three-spine stickleback by rainbow trout. *Ecology of Freshwater Fish* **20:** 308–314.

McCollum SA, Van Buskirk J. 1996. Costs and benefits of a predator-induced polyphenism in the gray treefrog *Hyla chrysoscelis*. *Evolution* **50:** 583–593.

McPhail JD. 1963. Geographic variation in North American nine-spine sticklebacks, *Pungitius pungitius*. *Journal of the Fisheries Research Board of Canada* **20:** 27–44.

Mobley KB, Lussetti D, Johansson F, Englund G, Bokma F. 2011. Morphological and genetic divergence in Swedish postglacial stickleback (*Pungitius pungitius*) populations. *BMC Evolutionary Biology* **11:** 287.

Moodie GEE. 1972. Predation, natural selection and adaptation in an unusual three-spine stickleback. *Heredity* **28:** 155–167.

Moran N. 1992. The evolutionary maintenance of alternative phenotypes. *The American Naturalist* **139:** 971–989.

Murtaugh PA. 2009. Performance of several variable-selection methods applied to real ecological data. *Ecology Letters* **12:** 1061–1068.

Nilsson PA, Brönmark C, Pettersson LB. 1995. Benefits of a predator-induced morphology in crucian carp. *Oecologia* **104:** 291–296.

Noonburg EG, Nisbet RM. 2005. Behavioural and physiological responses to food availability and predation risk. *Evolutionary Ecology* **7:** 89–104.

Nosil P, Crespi BJ. 2006. Experimental evidence that predation promotes divergence in adaptive radiation. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 9090–9095.

Östlund-Nilsson S, Mayer I, Huntingford FA. 2007. *Biology of the three-spined stickleback*. Boca Raton, FL: CRC Press.

Parsons KJ, Robinson BW. 2007. Foraging performance of diet-induced morphotypes in pumpkinseed sunfish (*Lepomis gibbosus*) favours resource polymorphism. *Journal of Evolutionary Biology* **20:** 673–684.

Pauwels K, Stoks R, De Meester L. 2010. Enhanced anti-predator defence in the presence of food stress in the water flea *Daphnia magna*. *Functional Ecology* **24:** 322–329.

Pfennig DW, Wund MA, Snell-Rood EC, Cruickshank T, Schlichting CD, Moczek AP. 2010. Phenotypic plasticity's impacts on diversification and speciation. *Trends in Ecology and Evolution* **25:** 459–467.

Reimchen TE. 1983. Structural relationships between spines and lateral plates in three-spine stickleback (*Gasterosteus aculeatus*). *Evolution* **37:** 931–946.

Reimchen TE. 1991. Trout foraging failures and the evolution of body size in stickleback. *Copeia* **1991:** 1098–1104.

Reimchen TE, Stinson EM, Nelson JS. 1985. Multivariate differentiation of parapatric and allopatric populations of three-spine stickleback in the Sangan River watershed, Queen Charlotte Islands. *Canadian Journal of Zoology* **63:** 2944–2951.

Richards CL, Bossdorf O, Muth NZ, Gurevitch J, Pigliucci M. 2006. Jack of all trades, master of some? On the role of phenotypic plasticity in plant invasions. *Ecological Letters* **9:** 981–993.

Roff DA. 1992. *The evolution of life histories*. New York: Chapman and Hall.

Rohlf FJ. 1999. Shape statistics: procrustes superimpositions and tangent spaces. *Journal of Classification* **16:** 197–223.

Rohlf FJ. 2006. *TPS software series*. Distributed by the author. Stony Brook, NY: Department of Ecology and Evolution, State University of New York.

Rohlf FJ, Slice DS. 1990. Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Zoology* **39:** 40–59.

Schluter D. 1996. Adaptive radiation along genetic lines of least resistance. *Evolution* **50:** 1766–1774.

Shapiro MD, Bell MA, Kingsley DM. 2006. Parallel genetic origins of pelvic reduction in vertebrates. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 13753–13758.

Shapiro MD, Summers BR, Balabhadra S, Aldenhoven JT, Miller AL, Cunningham CB, Bell MA, Kingsley DM. 2009. The genetic architecture of skeletal convergence and sex determination in nine-spine sticklebacks. *Current Biology* **19:** 1140–1145.

Sharpe DMT, Räsänen K, Berner D, Hendry AP. 2008. Genetic and environmental contributions to the morphology of lake and stream stickleback: implications for gene flow and reproductive isolation. *Evolutionary Ecology Research* **10:** 849–866.

Shimada Y, Shikano T, Kuparinen A, Gonda A, Leinonen T, Merilä J. 2011. Quantitative genetics of body size and timing of maturation in two nine-spined stickleback (*Pungitius pungitius*) populations. *PLoS ONE* **6:** e28859.

Spoljaric MA, Reimchen TE. 2008. Habitat-dependent reduction of sexual dimorphism in geometric body shape of Haida Gwaii three-spine stickleback. *Biological Journal of the Linnean Society* **95:** 505–516.

Stemberger RS, Gilbert JJ. 1984. Spine development in the rotifer *Keratella cochlearis*: induction by cyclopoid copepods and *Asplanchna*. *Freshwater Biology* **14:** 639–647.

Teplitsky C, Plenet S, Lena JP, Mermet N, Malet E, Joly P. 2005. Escape behaviour and ultimate causes of specific induced defences in an anuran tadpole. *Journal of Evolutionary Biology* **18:** 180–190.

Tollrian R. 1995. Predator-induced morphological defenses: costs, life history shifts, and maternal effects in *Daphnia pulex*. *Ecology* **76:** 1691–1705.

Tollrian R, Harvell CD. 1999. *The ecology and evolution of inducible defenses*. Princeton, NJ: Princeton University Press.

Välimäki K, Herczeg G. 2012. Ontogenetic and evolutionary effects of predation and competition on nine-spined stickleback (*Pungitius pungitius*) body size. *Journal of Animal Ecology* **81:** 851–857.

Van Buskirk J, Schmidt BR. 2000. Predator-induced phenotypic plasticity in larval newts: trade-offs, selection, and variation in nature. *Ecology* **81:** 3009–3028.

Van Buskirk J, Steiner UK. 2009. The fitness costs of developmental canalization and plasticity. *Journal of Evolutionary Biology* **22:** 852–860.

Vaughn D. 2007. Predator-induced morphological defenses in marine zooplankton: a larval case study. *Ecology* **88:** 1030–1039.

Waddington CH. 1953a. The 'Baldwin effect', 'genetic assimilation' and 'homeostasis'. *Evolution* **7:** 386–387.

Walker JA. 1997. Ecological morphology of lacustrine three-spine stickleback *Gasterosteus aculeatus* L. (*Gasterosteidae*) body shape. *Biological Journal of Linnean Society* **61:** 3–50.

Walker JA, Bell MA. 2000. Net evolutionary trajectories of body shape evolution within a microgeographic radiation of three-spine sticklebacks (*Gasterosteus aculeatus*). *Journal of Zoology* **252:** 293–302.

Walker JA, Ghalambor CK, Griset OL, McKenney D, Reznick DN. 2005. Do faster starts increase the probability of evading predators? *Functional Ecology* **19:** 808–815.

Webb PW. 1983. Speed, acceleration and manoeuvrability of two teleost fishes. *Journal of Experimental Biology* **102:** 115–122.

West-Eberhard MJ. 2003. *Developmental plasticity and evolution*. Oxford: Oxford University Press.

Wisenden BD, Chivers DP. 2006. The role of public chemical information in anti-predator behaviour. In: Ladich F, Collins SP, Moller P, Kapoor BG, eds. *Communication in fishes*. Enfield: Science Publisher, 259–278.

Wund MA, Baker JA, Clancy B, Golub JL, Foster SA. 2008. A test of the 'flexible stem' model of evolution: ancestral plasticity, genetic accommodation, and morphological divergence in the three-spine stickleback radiation. *American Naturalist* **172:** 449–462.

Wund MA, Valena S, Wood S, Baker JA, Street M. 2012. Ancestral plasticity and allometry in three-spine stickleback reveal phenotypes associated with derived, freshwater ecotypes. *Biological Journal of the Linnean Society* **105:** 573–583.

Young TP, Stanton ML, Christian CE. 2003. Effects of natural and simulated herbivory on spine lengths of *Acacia drepanolobium* in Kenya. *Oikos* **101:** 171–179.

Zelditch ML, Swiderski D, Sheets HD, Fink WL. 2004. *Geometric morphometrics for biologists: a primer*. New York: Elsevier academic press.

Ziuganov VV, Zotin AA. 1995. Pelvic girdle polymorphism and reproductive barriers in the nine-spine stickleback *Pungitius pungitius* (L.) from north-west Russia. *Behaviour* **132:** 1095–1105.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Summaries of the general linear mixed models on body shape variation captured by the second, third and fourth relative warps.

Figure S1. Visualization for all main effects in the general linear mixed models of the relative warps two (a–d), three (e–h) and four (i–l) and for the non-significant terms in general linear mixed models run on PC1 (m–n).

Sexual dimorphism in nine-spined stickleback (*Pungitius pungitius*) body shape and armour

Mirva Turtiainen[1], Gábor Herczeg[1,2], Juha Merilä[1,†]

[1] *Ecological Genetics Research Unit, Department of Biosciences, PO Box 65, FI-00014 University of Helsinki, Finland*

[2] *Present address: Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/C, Budapest, H-1117, Hungary*

[†]Author to whom correspondence should be addressed. Tel: +358-40-8374165; email: juha.merila@helsinki.fi

Abstract

Nine-spined stickleback (*Pungitius pungitius*) show considerable population variation in their morphology: individuals from marine and lake (high predation) populations have streamlined bodies and fully developed bony armour while individuals from pond (low predation) populations have deep bodies and reduced (or absent) bony armour. Here, we investigated sexual dimorphism (SD) in body shape and bony armour in 11 Fennoscandian nine-spined stickleback populations with special focus in comparison of the morphologically extreme marine and pond habitats. Our results show that females have relatively shallow bodies with long caudal peduncles and small heads, whereas males are characterized by deep bodies, large heads and short caudal peduncles. Females tended to be more heavily armoured than males, having more anterior and posterior lateral plates in addition to longer pelvic girdles and pelvic spines. Although all populations displayed the same direction of SD, the degree of SD varied significantly among populations, independently of habitat type. Possible selective forces leading to the observed patterns are discussed.

Keywords: body armour; body shape; *Pungitius pungitius*; sexual dimorphism

Introduction

Sexual dimorphism (SD) is of common occurrence in a wide range of animal taxa, and thought to reflect sex-specific differences in sexual or natural selection, or both (Darwin, 1874; Slatkin, 1984; Hedrick & Temeles, 1989). SD is not limited only to differences in sexual organs and secondary sexual traits, but it occurs also in other traits such as body size, body shape, feeding apparatus and behaviour (e.g. Andersson, 1994). These sexual differences in ecologically important traits are only indirectly or not at all related to reproduction, but often coupled with foraging differences between the sexes (Shine, 1989). The role of ecological factors in the evolution of SD has been extensively debated but remained often unclear (Slatkin, 1984; Shine, 1989). Ecological drivers underlying evolution of SD can stem from sex differences in habitat use, exposure to predators, diet or intraspecific competition between the sexes (Slatkin, 1984). Also differences in reproductive roles between the sexes can select for sex-specific differences in external and internal morphology (Casselman & Schulte-Hostedde, 2004). However, as compared to amount of work done with sexual size dimorphism (SSD) (e.g. Abouheif & Fairbairn, 1997; Kraushaar & Blanckenhorn, 2002; Blanckenhorn, 2005; Young, 2005; Herczeg et al., 2010a), SD in other phenotypic aspects, such as body shape, have been investigated much more seldom (Bonnet, 2001; but see: Kitano et al. 2007; Leinonen et al. 2010a).

The nine-spined stickleback (*Pungitius pungitius* Linnaeus, 1758) has a circumpolar distribution and occupies a variety habitats both in marine and freshwater environments (e.g. Bănărescu and Paepke, 2001; Östlund-Nilsson et al., 2007). It has gone through an adaptive radiation that has resulted extraordinary phenotypic variation between populations (Gross, 1979; Blown, 1992; Gonda et al. 2009; Herczeg et al. 2009; 2010a,b; Mobley et al. 2011). Its sister species, the three-spined stickleback (*Gasterosteus aculeatus* Linnaeus, 1758), has been studied extensively and sexual dimorphism of three-spined stickleback morphology is well known (e.g. Mori, 1984; Caldecutt & Adams, 1998; Reimchen & Nosil, 2004; Leinonen et al., 2006, 2010a; Kitano et al., 2007; Spoljaric & Reimchen, 2008; Aguirre & Akinpelu, 2010). In general, male three-spined sticklebacks have larger heads and mouths than females, why the latter are more streamlined and have longer caudal peduncles. However, the nine-spined stickleback currently became an increasingly used model organism as it not only offers a model to study adaptive divergence and local adaptation in general, but together with three-spined stickleback, it offers a model to test different questions related to parallel evolution between species (Shapiro et al. 2006; 2009). While variation in its general morphology has recently been described in detail (Herczeg et al. 2010b; Mobley et al. 2011), SD in its morphology has never been studied in any detail.

The aim of this study was to investigate occurrence and extent of sexual dimorphism (SD) in different populations of the nine-spined stickleback. Based on earlier work in SD in the three-spined stickleback (Kitano et al. 2007; Leinonen et al. 2010a) and an earlier study of nine-spined sticklebacks demonstrating similarity in habitat-based morphological shifts in

nine-spined and three-spined sticklebacks (Herczeg et al. 2010b), we hypothesized that (i) there should be SD in nine-spined stickleback body shape and armor, which is (ii) consistent across populations and (iii) similar to what was found in three-spined sticklebacks (i.e. males have larger heads and jaws than females, why the latter are longer and had longer caudal peduncles). To test these predictions, we compared body shape and armour among male and female nine-spined sticklebacks from 11 different populations covering much of Fennoscandia including both marine and freshwater habitats.

Materials and methods

Sampling

Adult fish were sampled from five marine, four lake and 10 pond locations (locations shown in Fig. 1) between 2006 and 2009 as described in Herczeg et al. (2010b). However, as we were unable to obtain a sufficient number of males from several marine and lake sites, the final sample usable for analyses of SD consisted of 11 populations, including two marine sites from the Baltic and Atlantic Seas and nine ponds from Finland, Sweden and Russia (surface area < 5.8 ha, Fig. 1; for details see Table 1 in Herczeg et al. 2010b). Sampling was carried out during the reproductive season between late May and early July with minnow traps, dip nets, and seine nets. Only sexually mature adult individuals were included into the analyses and the fish were sexed on site according to visual criteria.

Measurements

Collected fish were over-anaesthetized with MS222, and stored in 96% ethanol for approximately 2 months. The standard length was measured (from the tip of the mouth to the end of the tail base), and the fish were fixed in 4% formalin for a minimum of 2 weeks. The fish were then stained with Alizarin Red S (Pritchard & Schluter, 2001) in order to make the external bones visible. The left side of each fish was photographed with a Nikon D60 digital camera equipped with a high resolution Nikkor AF-S DX 18–55 mm f/3.5–5.6G ED lens (Nikon Corp., Tokyo, Japan) from a standard angle using a tripod. A ruler was placed in all photographs for scale.

Thirteen landmarks (see below) were marked with needles. Geometric morphometric analyses were used to determine variation in body shape. The landmarks (Fig. 2) were digitized from the images using tpsDig 2 v2.10 (Rohlf, 2006). The landmarks used were as follows (Fig. 2): 1, anterior tip of upper lip; 2, posterior edge of angular; 3, anterior tip of ectocoracoid; 4, posterior tip of ectocoracoid; 5, base of first anal ray on ventral midline (VML); 6, insertion of anal fin membrane on VML; 7, origin of caudal fin membrane on VML; 8, caudal border of hypural plate at lateral midline; 9, origin of caudal fin membrane on dorsal midline (DML); 10, insertion of dorsal fin membrane on DML; 11, base of the first dorsal fin ray on DML; 12, anterior junction of the first dorsal spine on DML; 13, posterior extent of the supraoccipital.

In addition, five other morphological traits were measured: 1, number of dorsal spines; 2, number of anterior lateral plates; 3, number of posterior lateral plates; 4, length of pelvic spine; 5, length of pelvic girdle. Anterior and posterior lateral plates were counted under a dissecting microscope (the separation of anterior and posterior plates was apparent because the fish used in the study had no plates in the midsection of their bodies), pelvic spine length was measured with a digital caliper, and pelvic girdle length was measured from the digital photographs with the aid of tpsDig 2, using an extra landmark at the caudal tip of the posterior process of the pelvic girdle on VML (point 'x' on Fig. 2) in the fish that had pelvic girdles. A more detailed description of the measurements is available in Herczeg et al. (2010b).

Statistical analyses

The digitized landmarks were superimposed by tpsRelw 1.46 (Rohlf, 2006). Partial warp scores were obtained with the same program from the superimposed specimens. Next, the relative warp analyses (on the partial warp scores) were conducted; this is equivalent to a principal component analysis (PCA) with equal weight of all partial warps at all spatial scales. The shape visualizations (Fig. 3) were also performed by tpsRelw 1.46.

First, we ran a multivariate general linear model (GLM) on all partial warps and uniform components, with population, sex and population × sex interaction as fixed factors and centroid size as a covariate. The latter measure is the square root of the sum of the squared distances from the centroid to each landmark (Bookstein, 1991), and has been reported as a reliable size measure (e.g. Leinonen et al., 2006). Second, we ran separate a univariate GLMs on the relative warps that captured more than 10% of the total variation (e.g. Berner et al., 2009), with sex and population as fixed factors and centroid size as a covariate.

The metric variables (length of pelvic girdle and spine) had to be corrected for body size, while the meristic ones (plate and spine numbers) not. Because there were individuals, and even whole populations, entirely missing some parts of the pelvic structure, using regression residuals or simply entering body size as a covariate into the models did not work. Hence, we used simple ratios between the length of the pelvic girdle or spine and standards body length (from the tip of the nose to the tailbase). We ran a PCA to collapse the original variables into a smaller set of independent variables and selected PCs that had an initial eigenvalue over one. Univariate GLMs were performed on the PC scores, with population, sex and population*sex as a fixed factors. All analyses were performed with SPSS 15.0 (SPSS Inc. Chicago, Illinois).

Results

The multivariate GLM on the partial warps and uniform components revealed that the sexes differed in a population specific way in overall shape ($N_{male} = 275$, $N_{female} = 275$, population : Wilk's $\alpha = 0.339$, $P < 0.001$; sex: Wilk's $\alpha = 0.927$, $P < 0.05$; population*sex: Wilk's $\alpha = 0.261$,

$P < 0.001$; centroid size: Wilk's $\alpha = 0.639$, $P < 0.001$; population*centroid size: Wilk's $\alpha =$ 0·406, $P < 0.001$).

The first three relative warps (RWs) described 73.6% of the variation in the data. The first RW described bending (a biologically uninformative artefact of fixation) being responsible for 39.99 % of the total variation (Fig. 3). This RW was not analysed any further. RW2 (20.15 % of total variation) was biologically informative, describing a gradient from fish with relatively small heads, long and shallow midbodies, and long caudal peduncles towards fish with relatively large heads, short and deep midbodies and short caudal peduncles. RW3 (13.46 % of total variation) was hard to interpret, but the main variation was in mouth orientation and caudal peduncle length.

The GLM on the RW2 revealed a significant population specific sex effect (sex: $F_{1,527}$=732.81, $P < 0.001$; population: $F_{10,527}$=75.46, $P < 0.001$; population*sex: $F_{10,527}$=6.05, $P < 0.001$; centroid size: $F_{1,527}$ = 62·96, $P < 0.001$). Females had shallow bodies with long caudal peduncles, whereas males were characterized by deep bodies, large heads and short caudal peduncles. The direction of SD was the same in every population but the level of divergence differed (Fig. 4).

The GLM on the RW3 revealed a population specific sex effect (population: $F_{1,527}$=63.74, $P <$ 0.001; sex: $F_{1,527}$=1.20, P= 0.274; population*sex: $F_{10,527}$=2.55, P = 0.005; centroid size: $F_{1,527}$ = 0.10, P =0.752). Despite the highly significant effect, the pattern itself is hard to interpret (Fig. 4).

The PCA run on the five morphological variables resulted in two PCs with an initial eigenvalue of greater than one. Based on the factor loadings (Table 1), the first PC (48.46% of total variance) described a gradient from fish with a low number of lateral plates (both anterior and posterior), together with small pelvic girdles and spines towards fish with a higher number of lateral plates and longer pelvic spines and girdles. The second PC (21.06% of total variation) revealed no significant biological information. The GLM on PC1 revealed a significant sex effect (sex: $F_{1,507}$=8.973, P = 0.003; population: $F_{1,507}$=273.05, $P < 0.001$; population*sex: $F_{10,507}$=1.813, P = 0.056). In most cases females had more armour having more lateral plates and a larger pelvic girdle and longer pelvic spine length than males (males: LS mean = -0.06, 95% CI = -0.11 – -0.01; females: LS mean = 0.045, 95% CI = -0.006 – 0.095). We did not detect sexual differences in PC2 (population: $F_{10,507}$=79.206, $P < 0.001$; sex: $F_{1,507}$=0.913, P = 0.072; population*sex: $F_{10,507}$=0.91, P = 0.51).

Discussion

The results of this study affirm that different populations of nine-spined stickleback share the same features of sexual dimorphism. Females have relatively shallow bodies with long caudal peduncles and long midbodies, small heads and jaw, whereas males are characterized by deeper and shorter midbodies, large heads and jaws in addition to short

caudal peduncles. Further, females tend to be more heavily armoured, having a relatively longer pelvic girdle, longer pelvic spines and more lateral plates than males. Even though there were among population differences in the degree of SD, this variance was habitat-independent and the direction of SD was the same in every population for both shape and armour traits. These size independent patterns of SD align with earlier findings (Herczeg et al. 2010a) demonstrating female biased sexual size dimorphism in this species. Hence, not only are female nine-spined sticklebacks larger than males, but they also posses more body armour and differently shaped body than males.

The sex differences in body shape could result from selection stemming from sex differences in reproductive roles, and concomitant differences in foraging habits. In a closely related and ecologically similar three-spined stickleback, it has been confirmed that as a consequence of differences in reproductive roles, the sexes use their environment differently during the breeding season. The males stay close to the nest, feeding on small invertebrates from the bottom and filtering the prey from the substrate while the females forage plankton in the limnetic zone (Reimchen & Nosil, 2004; Kitano et al., 2007; Aguirre & Akinpelu, 2010). Such different life cycle habits can lead to morphological adaptations to foraging differences between the sexes. Corresponding information on sex differences in feeding habits of the nine-spined stickleback is still lacking, but the breeding biology of the two species is highly similar (e.g. Östlund-Nilsson et al., 2007).

Sexual dimorphism in the head and mouth region seems to be a general feature in sticklebacks, and has been reported in the three-spined stickleback (e.g. Kitano et al., 2007; Aguirre & Akinpelu, 2010) and in Asian populations of *Pungitius tymensis*, and *P. sinensis* (e.g. Kobayashi, 1959; Chae & Yang, 1990). The extension of the jaw and head region in the nine-spined stickleback males could indicate an adaptation for feeding on large benthic invertebrates, implying natural selection for niche divergence between sexes. However, males also use their mouth when defending their territory from intruders during the breeding season and when they construct nest from plant materials (Wootton, 1984). Hence, relatively large heads and jaws in males as compared to females might also result from sexual selection.

The female nine-spined sticklebacks spend most of their time foraging in the open water to acquire energy for the production of eggs (Wootton, 1984). Fully developed armor of the females indicates an adaptation providing protection against gape-limited vertebrate predators that feed in the pelagic zone. The larger pelvic girdle may also be a primary defensive armor to protect the eggs in the abdominal cavity. The three-spined stickleback has similar sexual dimorphism in external bony armour and females tend to be generally more heavily armoured than males (Reimchen, 1980; Kitano et al., 2007; Leinonen et al. 2010a).

An interesting fact is that the observed sexual differences in shape and armor parallel exactly those observed in pond-marine shape and armor transition in this species (Herczeg

et al. 2010), as well as that seen between the three-spined stickleback marine and freshwater populations (for review, see Bell & Foster 1994). In other words, the elongated, small-headed and heavily armored females corresponds to the marine/pelagic, while the deep-bodied, large-headed, less armored males to the pond/benthic morphotype. This parallelism gives some support for the conjecture that sex differences in shape and armour may be driven by predation mediated selection. However, that this same general tendency is seen in all studied populations as well as in the close related three-spined stickleback might hint about existence of some type developmental or evolutionary constraints in acquiring shape and armour divergence among populations. In fact, Leinonen et al. (2010b) found that there are strong genetic correlations among armour and shape traits in three-spined sticklebacks. This suggests that the two sets of traits are not free to evolve independently, but selection on one (e.g. shape) may result in correlated responses in other (i.e. shape; Leinonen et al. 2010b).

Finally, it should be noted that the results presented in this study are based on material collected from the wild rather than from common garden experiments conducted under standardised conditions. This means that the documented SD may not reflect solely 'true' levels of SD in the study populations if the development of sexual differences is strongly affected by environment, or if selection (mortality) has shifted trait distributions before sampling (e.g. Leinonen et al. 2010a). While we think that the confirmatory common garden experiments needs to be conducted, we note that is unlikely that environmental effects or selection would have exerted any great influence on our results given the parallel patterns of sexual dimorphism in all study populations irrespectively of the habitat type.

In summary, there is clear SD in both body shape and armor in nine-spined sticklebacks. While populations differed in the level of SD, the direction of SD was the same in all populations and we found no indication of habitat specificity of SD. These patterns are in close resemblance to what was reported from three-spined sticklebacks earlier providing another example of similarity between the species. SD in three-spined stickleback morphology do have a genetic basis (Leinonen et al. 2010a), and it this is likely to be true also in the case of nine-spined sticklebacks. If so, the observed patterns of SD are most likely to result from sexual or natural selection acting differently on the two sexes. As our study is correlative by its nature, manipulative experiments will be needed to identify the selection forces causing or maintaining SD in body shape and armour in this species.

Acknowledgements

We thank Victor Berger, Jacquelin DeFaveri, Göran Englund, Lotta Kvarnström, Tuomas Leinonen, Daniel Lussetti, and Pirkko Siikamäki for their help in obtaining the samples. We thank the Oulanka Research Station (University of Oulu), the White Sea Biological Station (Russian Academy of Sciences) and Kristineberg Marine Biological Station (University of Göteborg) for their help with practicalities in the field. Our study was supported by the Academy of Finland (GH, JM) and by a BALTGENE-project from the BONUS+ programme.

References

Aguirre, W. E. & Akinpelu, O. (2010). Sexual dimorphism of head morphology in three-spined stickleback *Gasterosteus aculeatus*. *Journal of Fish Biology* **4**, 802–821.

Abouheif, E. & Fairbairn, D. J. (1997). A comparative analysis of allometry for sexual size dimorphism: assessing Rensch's rule. *American Naturalist* **149**, 540–562.

Andersson, M. (1994). Sexual Selection. Princeton, NJ: Princeton University Press.

Bănărescu P. M. & Paepke H-J. (2001). The Freshwater Fishes of Europe, Vol. 5/III. Wiebelsheim: AULA-Verlag.

Bell MA, Foster SA, eds. 1994. *The evolutionary biology of the threespine stickleback*. New York: Oxford Science.

Berner D., Grandchamp A-C & Hendry A. P. (2009). Variable progress toward ecological speciation in parapatry: stickleback across eight lake-stream transitions. *Evolution* **63**, 1740–1753.

Blanckenhorn W. U. (2005). Behavioural causes and consequences of sexual size dimorphism. *Ethology* **111**, 977–1016.

Blouw D. M. & Boyd D. J. (1992). Inheritance of reduction, loss, and asymmetry of the pelvis in *Pungitius pungitius* (ninespine stickleback). Heredity **68**, 33–42.

Bookstein F. L. (1991). *Morphometric Tools for Landmark Data: Geometry and Biology*. Cambridge: Cambridge University Press.

Caldecutt W. J., Bell M. A. & Buckland-Nicks J. A. (2001). Sexual dimorphism and geographic variation in dentition of threespine stickleback, *Gasterosteus aculeatus*. *Copeia* **2001**, 936–944.

Casselman S. J. & Schulte-Hostedde A. I. (2004). Reproductive roles predict sexual dimorphism in internal and external morphology of lake whitefish, *Coregonus clupeaformis*. *Ecology of Freshwater Fish* **13**, 217–222.

Chae, B. S. & Yang H. J. (1990). Sexual dimorphism in eightspine stickleback, *Pungitius sinensis*: Gasterosteidae. *Korean Journal of Zoology* **33**, 260–265.

Darwin C. (1874). The Descent of Man, and Selection in Relation to Sex. A. L. Burt, N.Y.

Gross H. P. (1979). Geographic variation in European ninespine Sticklebacks, *Pungitius pungitius*. *Copeia* **1979**, 405-412.

Hedrick A. V. & Temeles E. J. (1989). The evolution of sexual dimorphism in animals: hypotheses and tests. *Trends in Ecology and Evolution* **4**, 136–138.

Heins D. C., Johnson J. M. & Baker J. A. (2003). Reproductive ecology of the nine-spined stickleback from south-central Alaska. *Journal of Fish Biology* **63**, 1131–1143.

Herczeg G., Gonda A. & Merilä J. (2009). Evolution of gigantism in ninespine sticklebacks. *Evolution* **63**, 3190–3200.

Herczeg G., Gonda A. & Merilä J. (2010a). Rensch's rule inverted – female-driven gigantismin nine-spined stickleback *Pungitius pungitius*. *Journal of Animal Ecology* **79**, 581–588.

Herczeg G. Turtiainen M. & Merilä J. (2010b). Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution. *Biological Journal of Linnean Society* **101**, 403–416.

Kitano J., Mori S. & Peichel C. L. (2007). Sexual dimorphism in the external morphology of the threespine stickleback (*Gasterosteus aculeatus*). *Copeia* **2007**, 336–49.

Kobayashi H. (1959). Cross-experiments with three species of stickleback, *Pungitius pungitius* (L.), *Pungitius tymensis* (Nikolsky), and *Pungitius sinensis* (Guichenot), with special reference to their systematic relationship. *Journal of Hokkaido Gakugei University*, Section B **10,** 363–384.

Kraushaar U. & Blanckenhorn W. U. (2002). Population variation in sexual selection and its effects on size allometry in two dung fly species with contrasting sexual size dimorphism. *Evolution* **56**, 307–321.

Leinonen T., Cano J. M., Makinen H. & Merilä J. (2006). Contrasting patterns of body shape and neutral genetic divergence in marine and lake populations of threespine sticklebacks. *Journal of Evolutionary Biology* **19**, 1803–1812.

Leinonen T., J.M. Cano & J. Merilä (2010a). Genetic basis of sexual dimorphism in the threespine stickleback *Gasterosteus aculeatus*. Heredity 106: 218-227.

Leinonen T., J.M. Cano & J. Merilä (2010b). Genetics of body shape and armour variation in threespine sticklebacks. Journal of Evolutionary Biology 24: 206-218.

McLennan D. A. (1996). Integrating phylogenetic and experimental analyses: The evolution of male and female nuptial coloration in the stickleback fishes (Gasterosteidae). *Systematic Biology* **3**, 261–277.

Mobley K. B., Lussetti, D., Johansson, F., Englund, G. & Bokma, F. (2011). Morphological and genetic divergence in Swedish postglacial stickleback (*Pungitius pungitius*) populations. *BMC Evolutionary Biology* **11:**287

Mori S. (1984). Sexual dimorphism of the landlocked threespine stickleback *Gasterosteus aculeatus* microcephalus from Japan. *Japanese Journal of Ichthyology* **30**, 419–425.

Östlund-Nilsson S., Mayer I. & Huntingford F. A. (2007). Biology of The Three-spined Stickleback. Boca Raton: CRC Press.

Pritchard J. R. & Schluter D. (2001). Declining interspecific competition during character displacement: summoning the ghost of competition past. *Evolutionary Ecology Research* **3**, 209–220.

Reimchen T. E. (1980). Spine deficiency and polymorphism in a population of *Gasterosteus aculeatus:* an adaptation to predators? *Canadian Journal of Zoology* **58**, 1232—1244.

Reimchen T. E. & Nosil P. (2004). Variable predation regimes predict the evolution of sexual dimorphism in a population of threespine stickleback. *Evolution* **58**, 1274–1281.

Rohlf F. J. (2006). *TPS software series*. Distributed by the author. Stony Brook, NY: Department of Ecology and Evolution, State University of New York.

Rohlf F. J. & Slice D. S. (1990). Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Zoology* **39**, 40–59.

Shine R. (1989). Ecological causes for the evolution of sexual dimorphism: a review of the evidence. *Quarterly Review of Biology* **64**, 419–461.

Shikano T., Shimada Y., Herczeg G. & Merilä J. (2010). History *vs.* habitat type: explaining the genetic structure of European nine-spined stickleback (*Pungitius pungitius*) populations. *Molecular Ecology* **19**, 1147–1161.

Slatkin M. (1984). Ecological causes of sexual dimorphism. *Evolution* **38**, 622–30.

Spoljaric M. A. & Reimchen T. E. (2008). Habitat-dependent reduction of sexual dimorphism in geometric body shapes of Haida Gwaii threespine stickleback. *Biological Journal of the Linnean Society* **95**, 505–516.

Wootton R. J. (1976). The biology of The Sticklebacks. New York, NY: Academic Press.

Wootton R. J. (1984). Reproductive behavior of the sticklebacks. in *A Functional Biology of Sticklebacks*. (Wootton R. J., ed.) pp. 113-154, Berkeley, CA: University of California Press.

Young K. A. (2005). Life-history variation and allometry for sexual size dimorphism in Pacific salmon and trout. *Proceedings of the Royal Society B* **272**, 167–172.

Tables and Figures

Table 1. Principal Component Analysis ran on five morphological variables. Factor loadings and variance explained are shown.

	PC1	PC2
Number of dorsal spines	0.35	0.51
Number of anterior plate	0.71	0.51
Number of posterior plates	0.70	0.29
Pelvic spine length	0.86	-0.35
Pelvic girdle length	0.76	-0.57
% of variation explained	48.46	21.06

Figure 1. Map of Fennoscandia showing the locations of the sampled nine-spined stickleback (*Pungitius pungitius*) populations. For the abbreviations see Table 1 in Herczeg et al. (2010b).

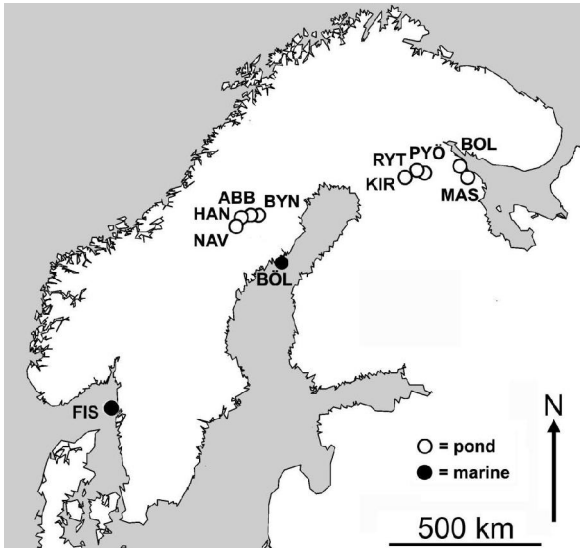


Figure 2. Illustration of the landmark positions for geometric morphometrics measurements: 1, anterior tip of upper lip; 2, posterior edge of angular; 3, anterior tip of ectocoracoid; 4, posterior tip of ectocoracoid; 5, base of first anal ray on ventral midline (VML); 6, insertion of anal fin membrane on VML; 7, origin of caudal fin membrane on VML; 8, caudal border of hypural plate at lateral midline; 9, origin of caudal fin membrane on dorsal midline (DML); 10, insertion of dorsal fin membrane on DML; 11, base of the first dorsal fin ray on DML; 12, anterior junction of the first dorsal spine on DML; 13, posterior extent of the supraoccipital; x, a landmark that was used separately from the others to measure pelvic girdle length.

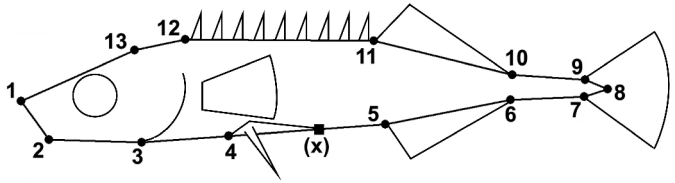


Figure 3. Results of the geometric morphometrics. The extreme phenotypes and the percentage of total variation covered by a given relative warp (RW) are shown. RW1 is describing bending and is assumed to be biologically uninformative.

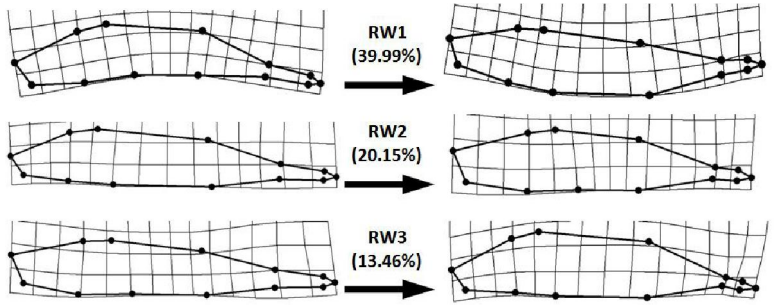
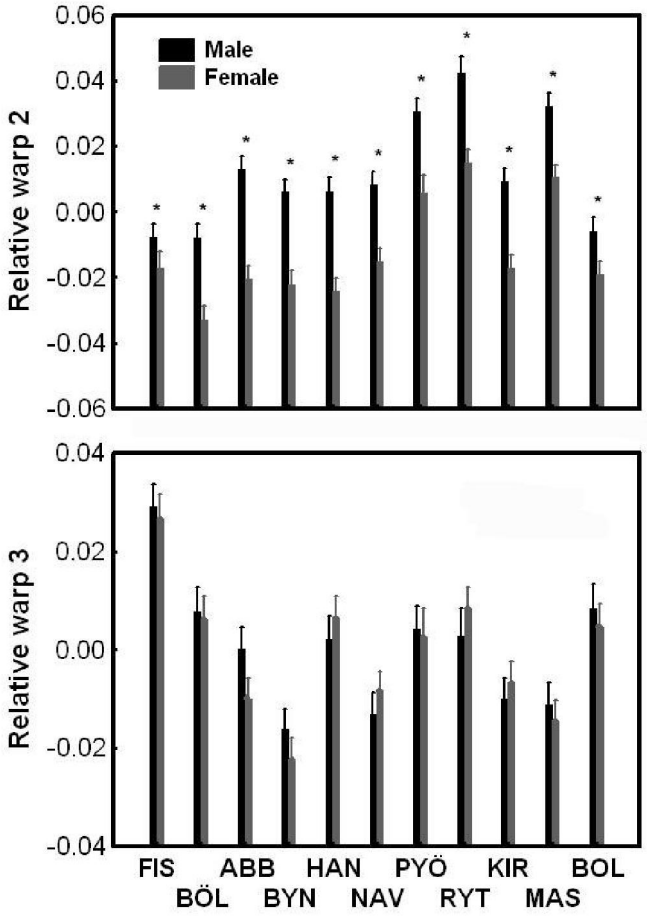


Figure 4. Sexual shape dimorphism in the different populations. Shape is described by relative warps (RWs), for details see Fig. 3. For the populations, see Fig. 1 and text. * denotes significant sexual dimorphism.



Intraspecific variation in energy storage traits in ecologically divergent nine-spined stickleback populations

Kaisa Välimäki[1], Gábor Herczeg[1,*], Juha Merilä[1]

[1] *Ecological Genetics Research Unit, Department of Biosciences, PO Box 65, FI-00014 University Helsinki, Finland*

[*] *Present address: Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/C, H-1117, Hungary*

Correspondence to: KV

E-mail: kaisa.valimaki@helsinki.fi

Running head: Intraspecific variation in energy storage.

Abstract

Energy storage is an important life-history trait which often trades off with growth or reproduction. Given that active foraging entails increased risk of predation and competition reduces resource availability, predation and competition can be expected to influence energy storage strategies in animals. Here, we tested if pond (low predation – high competition) and marine (high predation – low competition) nine-spined sticklebacks (*Pungitius pungitius*) exhibited different energy storage patterns in a factorial common garden experiment in which perceived predation risk and food supply were manipulated. We found that habitat of origin (pond *vs*. marine) had a strong effect on energy storage levels: pond fish had relatively higher lean body weight, but lower fatbody weight than marine fish. Marine fish tended to have also relatively heavier livers than pond fish, but mainly because of pronounced sexual dimorphism in ponds where males had smaller livers. Food treatment affected energy reserves in a habitat- and sex-specific ways. Perceived predation risk reduced lean body weight, but this effect was evident only in pond fish. In general, the results suggest existence of genetically based and possibly locally adapted energy storage strategies in sticklebacks.

Keywords: body mass, fat storage, energy allocation, geographic variation, liver size, predation risk

Introduction

Allocating surplus energy into storage is a general and wide-spread strategy among animals likely to have evolved to meet the demands exerted by fluctuations in energy intake opportunities (Reznick and Braun 1987; Mcnamara and Houston 1990; Varpe et al. 2009). Energy is often stored in form of lipids in muscle tissue, body cavity and liver, or as in form of glycogen in muscle and liver tissue (Chellappa et al. 1995). For many species, overwintering and reproduction are the critical and reoccurring periods of time during which organisms have to use their storages to fuel metabolism (Mcnamara and Houston 1990; Jönsson 1997; Biro et al. 2005; Varpe et al. 2009). However, energy allocation to storage is often conflicted with other energy-dependent processes such as predator avoidance (e.g. Veasey et al. 1998; Pérez-Tris et al. 2004) and somatic growth (Stearns, 1992; Roff, 1992). Thus, processes influencing investment to somatic growth are likely to be connected to investment to energy storage and *vice versa*.

Acquisition and expenditure of energy are important components of individual fitness (Roff 1992; Stearns 1992).Therefore energy storage strategies are expected to be under divergent natural selection in different environments resulting in intraspecific population differentiation. However, less attention has been paid towards biotic environmental factors. Predation is one of the most important environmental factors (Roff 1992) both on ontogenetic and evolutionary levels. So far research on energy storage has mainly focused on abiotic environmental gradients (*viz*. latitudinal, altitudinal or temperature) revealing marked within species divergence in energy storage (Schultz and Conover 1997; Finstad et al. 2009; Jönsson et al. 2009; Takahashi and Pauley 2010; Berg et al. 2011). How exactly do biotic environmental factors affect population differentiation in energy storage has been much less studied. It has been demonstrated experimentally that predation risk can induce phenotypic plasticity in energy storage (Lilliendahl 1998; Carrascal and Polo 1999; Martín and López 1999; Rands and Cuthill 2001; Pratt and Fox 2002; Garvey et al. 2004; Stoks et al. 2005). To date, we are not aware of any studies demonstrating population divergence in energy storage or energy storage plasticity in relation to population differences in the predation risk experienced in natural conditions.

To this end, nine-spined stickleback (*Pungitius pungitius*) - a small teleost fish that occurs in freshwater and marine systems throughout the northern hemisphere (Banarescu and Paepke 2001) – provides a good model: pond sticklebacks lacking sympatric piscine predators live longer, grow for an extended period of time and have delayed maturation. These traits are coupled with larger body size and increased reproductive output as compared to their marine conspecifics subject to piscine predation (Herczeg et al. 2009b, 2010a, 2012; Shimada et al. 2011; Ab Ghani et al. 2012). Pond and marine sticklebacks also differ in morphology, behavior and neural anatomy (Gonda et al. 2009; Herczeg et al. 2009b,c, 2010b; Trokovic et al. 2011). It has been suggested that in the absence of piscine predation and interspecific competition, success in intraspecific competition is the main driver of adaptive evolution resulting in the distinct pond phenotype (Herczeg et al. 2009b,c).

The aim of this study was to explore intraspecific energy storage variation induced by biotic interactions (predation and competition) using nine-spined stickleback as a model. We were interested in how evolutionary history of populations, individual experience during

development and sex affect energy allocation patterns. To study the question, we reared nine-spined sticklebacks originating from pond and marine populations in a factorial common garden experiment with manipulated food and perceived predation risk levels. Lean body weight, fatbody and liver weights were analysed after 34 weeks of development. The aim was to answer the following questions: (1) Has evolutionary history with predators influenced energy investment patterns? (2) Does predation risk or food availability induce plasticity in energy storage patterns? (3) Is there sexual dimorphism in energy storage levels? (4) How do evolutionary history, plasticity and sex interact? We expected that the competitive pond phenotype (Herczeg et al. 2009a,b, 2010a) will have lower energy reserves as they invest more in growth compared to their marine conspecifics (Herczeg et al. 2012), and that the predation-adapted marine sticklebacks will be more sensitive to predation risk than their pond conspecifics. We also predicted that the competition-adapted pond stickleback would be more sensitive to variation in food supply (Herczeg and Välimäki 2011; Välimäki et al. 2012).

Material & Methods

Breeding and experimental treatments

The fish used in this paper are the same as in in Herczeg and Välimäki (2011), Välimäki and Herczeg (2012), Välimäki et al. (2012). The full description of rearing and experimental procedures is given in the aforementioned references and only a brief summary of salient points is given here. Parental fish originated from two ponds (ABB =Abbortjärnen; PYÖ =Pyörelampi) and from four marine sites (BÖL=Bölesviken, HEL=Helsinki, NYK= Nyköping, KRI=Kristineberg, Fig. 1). Ponds were small (surface area < 5 ha) and free of piscine predators. Nine-spined stickleback was the only fish species in ABB, while a low number of whitefish (*Coregonus lavaretus*) has been introduced to PYÖ recently. Due to its diet whitefish are competitors to – not predators of –nine-spined sticklebacks. Marine sampling sites are characterized by diverse fish fauna including several gape-unlimited piscine predators.

Adult fish of the parental generation were caught in 2009 and kept in constant light conditions and fed in excess in University of Helsinki aquaculture facilities. At reaching reproductive stage, 6-10 full sib families per population were produced *in vitro* (ABB = 6, BÖL=6, HEL =8, KRI =10, NYK=8, PYÖ=7). When hatched fry reached free-swimming stage, they were put individually into 1.4 L containers in four Allentown Zebrafish Rack Systems (’racks‘; Aquaneering Inc., San Diego, CA, USA) each hosting 100 containers. Fish were randomly assigned into four treatment combinations (see below), aiming at equal population/family representation in every treatment combination. Fish were kept in constant +12°C and photoperiod was set to 14 hours light: 10 hours dark. Freshly hatched fry were fed with live brine shrimp nauplii (*Artemia* sp.) that were gradually replaced by bloodworms (Chironomid larvae) after 80 days of rearing.

The four racks were randomly assigned into two predation risk treatments and the containers within racks into two food treatments. For the predation treatment, a 150 L plastic tank was connected into every rack. Water - first filtered by biological and mechanical filters - circulated through these tanks and was then pumped to the individual containers within the rack. A pair of perches (*Perca fluviatilis*) was kept in two of the extra containers to provide the olfactory cues of predator. As a control, the two remaining containers were filled with water, but contained no perch. Perch is a common and abundant stickleback predator both in the Baltic Sea and the Fennoscandian freshwater systems (Koli 1990). Fish within families, predation treatments and racks were assigned randomly into high and low food treatments, being fed in excess two times per day *vs.* once per two days, respectively.

Sampling

A detailed description of the biochemical composition of nine-spined stickleback is lacking. However, in closely related three-spined stickleback (*Gasterosteus aculeatus*) carcass and liver are important stores of glycogen and lipids (Chellappa et al. 1989). Thus eviscerated body weight (hereafter lean body weight), weight of the liver, and the weight of the fatbody found in the body cavity were chosen as proxies of energy storage. At age of 34 weeks, fish were over-anesthetized using MS 222 (tricaine methanesulfonate). Digital images were taken from lateral side of each fish for measurement of standard length (taken from tip of the snout to the base of the tail) using a digital camera set on a tripod. Millimetre paper was used in every picture for scaling. Standard length was then measured from images using software TpsDig v.2.15 (Rohlf 2006). Fish were then weighed to a nearest mg using precision scale (XT 220A, Precisa, Dietikon, Switzerland). Finally, fish were dissected under stereomicroscope and their fat bodies and livers were carefully removed and weighed. Fresh lean body weight – defined as the eviscerated mass - was also measured. Fish and their organs were frozen in liquid nitrogen and stored in -80°C for further use. Sex of each individual was verified by gonadal inspection.

Statistical analyses

Lean body, fatbody and liver weights were analyzed using general linear mixed models (GLMMs) as implemented in PROC MIXED in SAS (Littell et al. 2006). The GLMMs were constructed with habitat (marine *vs.* pond), sex, food and predation treatments as fixed factors using population nested within habitat and family nested within population as random factors to account for non-independence in the data. We corrected for variation in fish size using following covariates: standard length for lean body weight analysis, and both standard length and body weight for analysing fatbody and liver weight. The latter correction was used because the standard length-body weight relationship differed significantly among populations ($F_{5,36}$=45.50, P < 0.0001). Full models consisted of all main effects and their interactions up to four-way level. We applied backward stepwise model selection based on the P < 0.05 criterion. This was done by starting with the highest level interaction progressing towards single effects while not removing any effect that was included in a significant higher level interaction. This approach is considered to be conservative compared to other model selection procedures (Murtaugh 2009). All statistical analyses were performed with SAS 9.2 (SAS Institute Inc., Cary, NC, USA) statistical software.

Results

Habitat, sex and food treatment, as well as the habitat × predator and habitat × sex interactions influenced variation in (relative) lean body weight (Table 1). Pond sticklebacks had a higher mean relative lean body weight than their marine conspecifics, but perceived threat of predation induced lowered body weight only in pond fish (Fig. 2a). Males had higher lean body weight than females, and this difference was more pronounced in pond than in marine fish (Fig. 2b). Furthermore, fish in the low food treatment were on average leaner than those in the high food treatment (Fig. 2c), and also population (Wald Z=1.25, P=0.053) and family (Wald Z=2.48, P=0.003) effects were significant (or close to).

In contrast to the lean body weight, variation in the relative fatbody weight was affected only by habitat and the food treatment × sex interaction (Table 1). Marine sticklebacks had about four times more fat than the pond fish (Fig. 3a). Food treatment did not affect relative male fatbody weight, but females had more fat in the high as compared to low food treatment (Fig. 3b). Also, habitat × sex interaction approached significance (P=0.058; Table 1), but the results did not show any clear patterns. Both population (Wald Z=1.13, P=0.0142) and family (Wald Z= 2.49, P=<0.0001) effects were significant.

Variation in the relative liver weight was affected by habitat, sex, food treatment and the habitat × sex interaction (Table 1). Fish in the high food treatment had relatively lighter livers than fish in the low food treatment (Fig. 4a). Marine sticklebacks had heavier livers than their pond conspecifics, and females had heavier livers than males, sexual dimorphism being stronger in ponds (Table 1, Fig 4b). Population and family effects were both significant (Population Wald Z=1.21, P=0.002; family Wald Z= 3.06, P=0.0001).

Discussion

The most salient finding of this study is the strong effect of habitat type on all tested energy storage variables. As indicated by relatively high lean body weight, pond sticklebacks appear to allocate more to functional tissues such as bone and muscle tissues, while marine sticklebacks appear to allocate more to energy reserves as indicated by their relatively high fat body and liver weights. Not surprisingly, food availability influenced energy reserves, but perceived predation risk affected only lean body weight. Sexual dimorphism in energy storage was also observed, albeit in habitat- and treatment-specific manner. In the following, we discuss these findings and their implications on our understanding of energy allocation and its dependency on populations' evolutionary history, sex and phenotypic plasticity.

The effects of evolutionary history

Nine-spined stickleback originating from piscine predator-free Fennoscandian ponds can reach giant sizes and high reproductive output through an extended period of growth and delayed maturation as compared to their marine or lake conspecifics (Herczeg et al. 2009a, 2010, 2012; Shikano et al. 2011; Nurul Ab Ghani et al. 2012). This life history divergence is suggested to be driven by intensified intraspecific competition under negligible predation and interspecific competition in ponds (see e.g.Herczeg et al. 2012). This inference is supported by observations that pond sticklebacks are more aggressive, risk-taking, and exhibit higher feeding activity as well as higher costs of group living than their marine conspecifics (Gonda et al. 2009; Herczeg et al. 2009b; Herczeg and Välimäki 2011). Assuming that the key to nine-spine stickleback success in ponds is to outgrow - and consequently - to outcompete and outreproduce conspecifics, one would expect pond sticklebacks to invest all energy available to growth before the first reproduction and hence minimize allocation to energy storage. In line with this expectation, we found that pond fish have considerably lower relative fatbody and liver weights than their marine conspecifics reared under identical conditions. Several studies have found that large fish have a higher body fat content than smaller fish (Schultz & Conover, 1997; Robards et al., 1999; Post & Parkinson, 2001; Sogard & Spencer, 2004), but this is obviously not the case in our study. These patterns of energy storage, coupled with faster growth in ponds (Shimada et al. 2011; Herczeg et al. 2012; Välimäki and Herczeg 2012) fit the pattern of life history divergence between pond and marine nine-spine sticklebacks.

We also found that pond sticklebacks have a higher relative lean body weight than marine fish. Lean body weight consists mainly of functional tissues (muscle and bone), but also glycogen and lipids can be stored in the carcass (Chellappa et al. 1989; Huntingford et al. 2001). Thus, variation in lean body weight can reflect both investments in energy storage and locomotive performance. Higher muscular mass can produce higher competitive performance (Casselman and Schulte-Hostedde 2004; Stahlschmidt et al. 2011) and be an advantage when levels of intraspecific competition are high, like in the pond systems of our study. On the other hand, higher muscle mass can improve escape performance for instance in crucian carps (Domenici et al. 2008) and decrease mortality caused by predation. If lean body weight indicates mainly investment on performance instead of energy storage, marine fish would be expected to invest significantly on it. However, for fish species with armour and spines, the role of quick startle response is often less important in predator avoidance (Andraso 1997; Godin 1997). Considering that the pond phenotype is aggressive, bold, and competitive (Herczeg et al. 2009b; Herczeg and Välimäki 2011), and that investment on lean body weight may improve competitive ability, it seems more likely that pond sticklebacks are storing energy in their muscles instead of fat bodies and liver. That said, we cannot distinguish between the alternatives without further studies on the carcass composition.

A considerable amount of research focusing on energy allocation patterns in different populations of fish has concentrated on variation over latitudinal or altitudinal gradients and to the energy storage for overwinter survival (Schultz and Conover 1997; Finstad et al. 2009; Jönsson et al. 2009; Takahashi and Pauley 2010; Berg et al. 2011). These studies have found that individuals from northern/higher altitude populations often have higher intrinsic growth rate and that they allocate more on energy storage than individuals from southern/lower altitude populations. Here the pond populations were the two northernmost populations, suggesting that latitudinal variation might contribute to the observed habitat specific differences. However, the observed energy storage patterns do not support this interpretation – even though the lean body weight of pond individuals was the highest, they had the smallest lipid storages and lowest liver weights.

Phenotypic plasticity in energy storage

In many fish species predation pressure causes strong trade-offs between somatic growth and energy storage (Sogard 1997; Post et al. 1999; Post and Parkinson 2001; Biro et al.

2006) and mortality is often directly dependent on fish size (Persson et al. 1996). One of the main aims of this experiment was to characterize how presence or absence of predators directly affects the energy storage patterns. We found surprisingly little evidence for predator mediated variation in energy storage levels. The only effect we found was that the predator presence decreased lean body weight, but this effect was only significant in pond populations. Three earlier studies have shown that perceived predation risk reduces energy reserves in fish. Juvenile walleyes (*Stizostedion vitreum*) had lower body weight and lower lipid content, but larger protein reserves, when reared in the presence of predators as compared to controls (Pratt and Fox 2002). Similarly, largemouth bass (*Micropterus salmoides*) gained mass and built up lipid reserves during overwintering in predator free experimental ponds, while they could only maintain the initial level of energy reserves in ponds where also predators were present (Garvey et al. 2004). Arctic charrs (*Salvelinus alpinus*) responded to olfactory cues from pikeperch (*Sander lucioperca*) with lowered body condition and water content, but elevated lipid content (Laakkonen 2006). Our expectation was that the marine fish should show stronger response to perceived predation than pond fish, because piscine predation is invariably absent in ponds (cf. Sultan & Spencer, 2002; Scheiner, 1993). In line with this expectation, marine sticklebacks show stronger predation-induced growth and body size responses to predator presence than pond sticklebacks (Välimäki and Herczeg 2012), but apparently not in terms of energy storage. We found that predator presence decreased lean body weight only in pond fish. This result resembles that of predator induced plasticity in brain architecture where also only pond fish responded (Gonda et al. 2012)). However, as the pattern in lean body weight was weak, the significance of this remains as yet unclear.

In terms of lean body weight, the effects of food treatment were as expected – fish in the high food treatment had on average higher lean body weights than those in low food treatments indicating better body condition in presence of abundant food. However, whether this better 'body condition' reflects larger energy reserves or more functional tissues, remains to be studied in the future. In contrast, liver weight responded to the high food treatment with a slight decrease. Liver stores both lipids and glycogen (Chellappa et al. 1989) and larger liver size should be indicative of larger energy reserves (Chellappa et al. 1995). In general, liver size has increased or remained at the same level as a response to the increased energy intake in feeding manipulation experiments (Allen and Wootton 1982; Pelletier et al. 1994; Hillestad and As 1998; Ali and Wootton 1999; Rideout et al. 2004). It is possible that liver size itself was unaffected by the food treatments, and it only appears smaller due to the increase in lean body weight.

Sexual dimorphism

Sexual dimorphism in energy storages is likely to reflect the differential investment in reproduction. Production of gametes is more demanding for females, whereas for males the cost relates to mate acquisition or as in sticklebacks, also to parental care (Bonnet, Shine, & Naulleau 1998; Casselman & Schulte-Hostedde 2004; Kokita & Mizota 2002; Santos, Amadio, & Ferreira 2010). Males therefore often invest on muscle mass to aid locomotion in aggressive encounters (Schulte-Hostedde et al. 2001; Casselman and Schulte-Hostedde 2004). In line with this, we found that male sticklebacks had higher relative lean body weight than females, even though in the nine-spined stickleback females are the larger sex (Herczeg et al. 2010a). The phenomenon appears to be common throughout the animal kingdom: males often have larger muscle mass reflecting the demands of increased physical performance required in mate acquisition (Bonnet et al. 1998; Schulte-Hostedde, Millar, & Gibbs 2002). While fish from both habitats showed the same pattern, it was somewhat more pronounced in ponds. Perhaps females are investing more to growth (in terms of sheer length) than males and this difference is stronger in ponds where growth is faster in general (Herczeg et al. 2012). Females should invest on organs that are important for food acquisition and processing, such as intestines and liver, and females are also expected to have a higher fat content to support energy demands of egg production (Bonnet et al. 1998; Casselman and Schulte-Hostedde 2004). In line with these expectations, we found females to have larger livers than males, but in marine populations both sexes had heavier livers than individuals in pond populations. In addition to energy storage and other functions, liver has important role in producing vitellogenin. Vitellogenin is processed to yolk proteins and larger liver size is connected to the more energy rich eggs (Henderson et al. 1996; Lambert and Dutil 1997; Dahle et al. 2003; Guijarro et al. 2003) which is a possible explanation for the sexual dimorphism in liver size. The larger reproductive output in pond females seems to be linearly related to their size (Ab Ghani et al. 2012). The sexual dimorphism in liver size is more pronounced in ponds similarly to sexual dimorphism in body size (Herczeg et al. 2010) corresponding with the hypothesis that life history divergence between the pond and marine sticklebacks is mainly driven by fecundity selection acting on females.

While both lean body and liver weight showed habitat specific sexual dimorphism, fatbody responded to the food treatment only in sex dependent manner. Males had equal relative fatbody weights in both treatments, while females showed a plastic response. Females had lower fatbody weight than males in low food treatment, and higher in high food treatment. This can reflect the sexual size dimorphism observed in nine-spined sticklebacks: females are the larger sex and thus are expected to invest more on somatic growth. Similar result was found in Yarrow's Spiny Lizard (*Sceloporus jarrovii*), where the smaller sex (females) had higher fat storages, especially under restricted feeding conditions (Cox et al. 2008). While we do not currently have any explanation for this pattern, it clearly demonstrates the environment-dependency of sexual dimorphism in important life history traits.

Conclusions

In summary, we found that the evolutionary history of populations, ontogenetic experience of individuals and sex all affected energy storage patterns in the nine-spined stickleback. Predation adapted marine sticklebacks maintained higher energy reserves than competition-adapted pond fish, which in turn were in better body condition in terms of length-corrected eviscerated body mass. The habitat-divergence in energy investment strategies can be understood in the light of ecological differences between habitats: in marine environments and under high predation risk, sticklebacks grow to small size, reach it quickly and mature early, whereas in the piscine predation free ponds fish grow as large as possible to gain competitive advantage. Predation induced slight decrease in pond fish body size and available food affected the energy reserves. Sexual dimorphism was strong, males had generally larger relative lean body weight while females had larger livers, and sex also often interacted with habitat or the treatments. Our study pioneers in demonstrating that biotic effects like predation and competition can be important evolutionary drivers of local adaptations in energy storage patterns and importantly that ontogenetic environment and sex can modify these effects.

Acknowledgements

Jacquelin DeFaveri, Göran Englund, Jenni Hämäläinen, Lotta Kvarnemo, Tuomas Leinonen, Pia Rännänen and Pirkko Siikamäki helped to obtain the parental samples. Mirva Turtiainen, Linda Uoti, Maarit Haataja and Marika Lilja helped with fish maintenance, thank you all for your efforts. Our work was supported by Academy of Finland (GH, # 128716; JM, #134728 & #129662), Finnish Graduate School in Population Genetics, Eemil Aaltonen Foundation and Georg and Ella Ehrnrooth Foundation (KV).The experiment was conducted under the license of the Finnish National Animal Experiment Board (Licence number STH223A).

Literature

Ab Ghani, I. N., J. Merilä and G. Herczeg. 2012. Body size divergence in nine-spined sticklebacks: disengtangling additive genetic and maternal effects. Biological Journal of the Linnean Society in press DOI: 10.1111/j.1095–8312.2012.01956.x.

Ali, M. and R. J. Wootton. 1999. Effect of variable food levels on reproductive performance of breeding female three-spined sticklebacks. Journal of Fish Biology 55:1040–1053.

Allen, J. R. M. and R. J. Wootton. 1982. Effect of food on the growth of carcase, liver and ovary in female *Gasterosteus aculeatus* L. Journal of Fish Biology 21:537–547.

Andraso, G. M. 1997. A comparison of startle response in two morphs of the brook stickleback (*Culaea inconstans*): Further evidence for a trade-off between defensive morphology and swimming ability. Evolutionary Ecology 11:83–90.

Banarescu, P. M. and H. J. Paepke. 2001. The Freshwater Fishes of Europe, Vol 5/III. AULA-Verlag, Wiebelsheim.

Berg, O. K., G. Rød, O. Solem and A. G. Finstad. 2011. Pre-winter lipid stores in brown trout Salmo trutta along altitudinal and latitudinal gradients. Journal of Fish Biology 79:1156–1166.

Biro, P. A., M. V. Abrahams, J. R. Post and E. A. Parkinson. 2006. Behavioural trade-offs between growth and mortality explain evolution of submaximal growth rates. Journal of Animal Ecology 75:1165–1171.

Biro, P. A., J. R. Post and M. V. Abrahams. 2005. Ontogeny of energy allocation reveals selective pressure promoting risk-taking behaviour in young fish cohorts. Proceedings of the Royal Society B 272:1443–1448.

Bonnet, X., R. Shine and G. Naulleau. 1998. Sexual dimorphism in snakes: different reproductive roles favour different body plans. Proceedings of the Royal Society B 265:179–183.

Carrascal, L. and V. Polo. 1999. Coal tits, *Parus ater*, lose weight in response to chases by predators. Animal behaviour 58:281–285.

Casselman, S. J. and A. I. Schulte-Hostedde. 2004. Reproductive roles predict sexual dimorphism in internal and external morphology of lake whitefish , *Coregonus clupeaformis*. Ecology of Freshwater Fish 13:217–222.

Chellappa, S., F. A. Huntingford, R. H. C. Strang and R. Y. Thomson. 1989. Annual variation in energy reserves in male three-spined stickleback (Gasterosteus aculeatus) L. (Pisces, Gasterosteidae). Journal of Fish Biology 35:275–286.

Cox, R. M., M. M. Barrett and H. B. John-Alder. 2008. Effects of food restriction on growth, energy allocation, and sexual size dimorphism in Yarrow's Spiny Lizard, *Sceloporus jarrovii*. Canadian Journal of Zoology 86:268–276.

Dahle, R., G. L. Taranger, Ø. Karlsen, O. S. Kjesbu and B. Norberg. 2003. Gonadal development and associated changes in liver size and sexual steroids during the reproductive cycle of captive male and female Atlantic cod (*Gadus morhua* L.). Comparative Biochemistry and Physiology A 136:641–653.

Domenici, P., H. Turesson, J. Brodersen and C. Brönmark. 2008. Predator-induced morphology enhances escape locomotion in crucian carp. Proceedings of the Royal Society B 275:195–201.

Finstad, A. G., O. K. Berg, T. Forseth, O. Ugedal and T. F. Naesje. 2009. Adaptive winter survival strategies: defended energy levels in juvenile Atlantic salmon along a latitudinal gradient. Proceedings of the Royal Society B 277:1113–1120.

Garvey, J. E., K. G. Ostrand and H. Wahl, David. 2004. Energetics, predation, and ration affect size-dependent growth and mortality of fish during winter. Ecology 85:2860–2871.

Godin, J.-G. J. 1997. Evading predators. Pp. 191–236 *in* J.-G. J. Godin, ed. Behavioural ecology of teleost fishes. Oxford University Press, Oxford.

Gonda, A., G. Herczeg and J. Merilä. 2009. Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (Pungitius pungitius) brain. Proceedings of the Royal Society B 276:2085–2092.

Guijarro, A. I., M. A. Lopez-Patino, M. L. Pinillos, E. Isorna, N. DePedro, A. L. Alonso-Gomez, M. Alonso-Bedate and M. J. Delgado. 2003. Seasonal changes in haematology and metabolic resources in the tench. Journal of Fish Biology 62:803–815.

Henderson, B. A., J. L. Wong and S. J. Nepszy. 1996. Reproduction of walleye in Lake Erie: allocation of energy. Canadian Journal of Fisheries and Aquatic Sciences 53:127–133.

Herczeg, G., A. Gonda, A. Kuparinen and J. Merilä. 2012. Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? Evolutionary Ecology 26:109–122.

Herczeg, G., A. Gonda and J. Merilä. 2009a. Evolution of gigantism in nine-spined sticklebacks. Evolution 63:3190–3200.

Herczeg, G., A. Gonda and J. Merilä. 2009b. Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). Journal of Evolutionary Biology 22:544–552.

Herczeg, G., A. Gonda and J. Merilä. 2010a. Rensch's rule inverted--female-driven gigantism in nine-spined stickleback Pungitius pungitius. Journal of Animal Ecology 79:581–588.

Herczeg, G., A. Gonda and J. Merilä. 2009c. The social cost of shoaling covaries with predation risk in nine-spined stickleback, *Pungitius pungitius*, populations. Animal Behaviour 77:575–580.

Herczeg, G., M. Turtiainen and J. Merilä. 2010b. Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): Biological Journal of the Linnean Society 101:403–416.

Herczeg, G. and K. Välimäki. 2011. Intraspecific variation in behaviour: effects of evolutionary history, ontogenic experience and sex. Journal of Evolutionary Biology 24:2434–2444.

Hillestad, M. and F. J. B. As. 1998. Long-term effects of dietary fat level and feeding rate on growth, feed utilization and carcass quality of Atlantic salmon. Aquaculture Nutrition 4:89–97.

Huntingford, F. A., S. Chellappa, A. C. Taylor and R. H. C. Strang. 2001. Energy reserves and reproductive investment in male three-spined sticklebacks, *Gasterosteus aculeatus*. Ecology of Freshwater Fish 10:111–117.

Jönsson, K. I. 1997. Capital and income breeding as alternative tactics of resource use in reproduction. Oikos 78:57–66.

Jönsson, K. I., G. Herczeg, R. B. O'Hara, F. Söderman, A. F. H. Ter-Schure, P. Larsson and J. Merilä. 2009. Sexual patterns of prebreeding energy reserves in the common frog *Rana temporaria* along a latitudinal gradient. Ecography 32:831–839.

Koli, L. 1990. Suomen Kalat. Werner Söderström, Helsinki.

Laakkonen, M. V. M. 2006. The effects of long-term predator exposure on body composition and condition of young Arctic charr (*Salvelinus alpinus*). Annales Zoologici Fennici 263–270.

Lambert, Y. and J. Dutil. 1997. Can simple condition indices be used to monitor and quantify seasonal changes in the energy reserves of cod (Gadus morhua)? Canadian Journal of Fisheries and Aquatic Sciences 54:104–112.

Lilliendahl, K. 1998. Yellowhammers get fatter in the presence of a predator. Animal behaviour 55:1335–1340.

Littell, R. C., G. A. Milliken, W. W. Stroup and W. R. D. 2006. SAS System for Mixed Models. SAS Institute Inc., Cary NC.

Martín, J. and P. López. 1999. An experimental test of the costs of antipredatory use in refuge the wall lizard, Podarcis murals. Oikos 84:499–505.

Mcnamara, J. M. and A. I. Houston. 1990. The value of fat reserves and the tradeoff between starvation and predation. Acta Biotheretica 38:37–61.

Pelletier, D., J. Dutil, P. Blier and H. Guderley. 1994. Relation between growth rate and metabolic organization of white muscle , liver and digestive tract in cod , *Gadus morhua*. Journal of Comparative Physiology B 164:179–190.

Post, J. R. and E. A. Parkinson. 2001. Energy allocation strategy in young fish: allometry and survival. Ecology 82:1040–1051.

Post, J. R., E. A. Parkinson and N. T. Johnston. 1999. Density-dependent processes in structured fish populations: interaction strengths in whole-lake experiments. Ecological Monographs 69:155–175.

Pratt, T. C. and M. G. Fox. 2002. Influence of predation risk on the overwinter mortality and energetic relationships of young-of-year Walleyes. Transactions of the American Fisheries Society 131:885–898.

Pérez-Tris, J., J. A. Díaz and J. L. Tellería. 2004. Loss of body mass under predation risk: cost of antipredatory behaviour or adaptive fit-for-escape? Animal Behaviour 67:511–521.

Rands, S. A. and I. C. Cuthill. 2001. Separating the effects of predation risk and interrupted foraging upon mass changes in the blue tit Parus caeruleus. Proceedings of the Royal Society B 268:1783–1790.

Reznick, D. N. and B. Braun. 1987. Fat cycling in the mosquitofish (Gambusia affinis): fat storage as a reproductive adaptation. Oecologia 73:401–413.

Rideout, R. M., E. A. Trippel and M. K. Litvak. 2004. Relationship between sperm density , spermatocrit , sperm motility and spawning date in wild and cultured haddock. Journal of Fish Biology 65:319–332.

Robards, M. D., J. A. Anthony, G. A. Rose and J. F. Piatt. 1999. Changes in proximate composition and somatic energy content for Pacific sand lance (*Ammodytes hexapterus*) from Kachemak Bay, Alaska relative to maturity and season. Journal of Experimental Marine Biology and Ecology 242:245–258.

Roff, D. A. 1992. The Evolution of Life Histories. Chapman & Hall, New York.

Rohlf, F. J. 2006. TPS software series. Distributed by the author. Department of Ecology and Evolution, State University of New York, Stony Brook, NY.

Scheiner, S. M. 1993. Genetics and evolution of phenotypic plasticity. Annual Review of Ecology and Systematics 24:35–68.

Schulte-Hostedde, A. I., J. S. Millar and G. J. Hickling. 2001. Sexual dimorphism in body composition of small mammals. Canadian Journal of Zoology 79:1016–1020.

Schultz, E. T. and D. O. Conover. 1997. Latitudinal differences in somatic energy storage: adaptive responses to seasonality in an estuarine fish (Atherinidae: *Menidia menidia*). Oecologia 109:516–529.

Shimada, Y., T. Shikano, A. Kuparinen, A. Gonda, T. Leinonen and J. Merilä. 2011. Quantitative genetics of body size and timing of maturation in two nine-spined stickleback (*Pungitius pungitius*) populations. PloS One 6:e28859.

Sogard, S. M. 1997. Size selective mortality in the juvenile stages of teleost fishes: a review. Bulletin of Marine Science 60:1129–1157.

Sogard, S. M. and M. L. Spencer. 2004. Energy allocation in juvenile sablefish: effects of temperature , ration and body size. Journal of Fish Biology 64:726–738.

Stahlschmidt, Z. R., J. R. Davis and D. F. Denardo. 2011. Sexual variation in assimilation efficiency: its link to phenotype and potential role in sexual dimorphism. Journal of Comparative Physiology B 181:383–389.

Stearns, S. C. 1992. The Evolution of Life Histories. Oxford University Press, Oxford.

Stoks, R., M. De Block and M. a. McPeek. 2005. Alternative growth and energy storage responses to mortality threats in damselflies. Ecology Letters 8:1307–1316.

Sultan, S. E. and H. G. Spencer. 2002. Metapopulation structure favors plasticity over local adaptation. American Naturalist 160:271–283.

Takahashi, M. K. and T. K. Pauley. 2010. Resource allocation and life history traits of *Plethodon cinereus* at different elevations. The American Midland Naturalist 163:87–94.

Trokovic, N., G. Herczeg, R. J. Scott McCairns, I. Nurul Ab Ghani and J. Merilä. 2011. Intraspecific divergence in the lateral line system in the nine-spined stickleback (*Pungitius pungitius*). Journal of Evolutionary Biology 24:1546–1558.

Varpe, Ø., C. Jørgensen, G. A. Tarling and Ø. Fiksen. 2009. The adaptive value of energy storage and capital breeding in seasonal environments. Oikos 118:363–370.

Veasey, J., N. Metcalfe and D. Houston. 1998. A reassessment of the effect of body mass upon flight speed and predation risk in birds. Animal behaviour 56:883–889.

Välimäki, K. and G. Herczeg. 2012. Ontogenetic and evolutionary effects of predation and competition on nine-spined stickleback (*Pungitius pungitius*) body size. Journal of Animal Ecology 81:859–867.

Välimäki, K., G. Herczeg and J. Merilä. 2012. Morphological antipredator defences in the nine-spined stickleback: constitutive, induced or both? Biological Journal of the Linnean Society in press.

Tables & Figures

Table 1 Results from the general linear mixed models (GLMM) on lean body weight, fatbody weight and liver weight of nine-spined stickleback originating from marine *vs.* pond habitats and reared under different predation and food treatments. Backward stepwise model selection was applied (see text). Nonsignificant results are shown as seen at the one-by-one back-substitution to the final model. Ndf and ddf denote numerator and denominator degrees of freedom, respectively.

	LEAN BODY		FATBODY		LIVER	
Variable	F	Ndf/ddf	F	Ndf/ddf	F	Ndf/ddf
Habitat	13.02	(1/4)**	26.43	(1/4.65)***	15.47	(1/4.65)**
Sex	38.36	(1/251***)	0.24	(1/280)	83.61	(1/280)***
Predator	3.42	(1/251)	1.01	(1/266)	0.12	(1/271)
Food	31.65	(1/251)***	3.94	(1/266)*	11.57	(1/270***)
Habitat*predator	4	(1/251)*	0.58	(2/265)	0.2	(2/271)
Habitat*food	0.96	(1/250)	0.31	(1/260)	0.26	(1/266)
Habitat*sex	4.72	(1/251)*	2.48	(1/268)	47.85	(1/272)***
Predator*food	1.35	(1/250)	1.12	(2/263)	0.11	(2/266)
Predator*sex	0.74	(1/250)	1.8	(2/270)	1.24	(2/272)
Food*sex	1.1	(1/250)	5.34	(1/266)*	2.23	(1/268)
Habitat*predator*food	1.19	(3/248)	0.56	(5/259)	0.34	(5/264)
Habitat*predator*sex	0.39	(2/249)	1.43	(5/268)	0.8	(4/273)
Habitat*food*sex	0.68	(3/248)	1.15	(3/265)	1.25	(3/267)
Predator*food*sex	0.89	(4/247)	2.17	(4/267)	1.38	(5/266)
Habitat*predator*food*sex	0.81	(9/242)	1.17	(11/259)	1	(11/262)
Standard length	437.89	(1/251)***	0.01	(1/288)	9.8	(1/291)***
Body weight			55.79	(1/286)	411.17	(1/292)***

*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 1. Map of the populations used to collect the parental generation for this experiment.

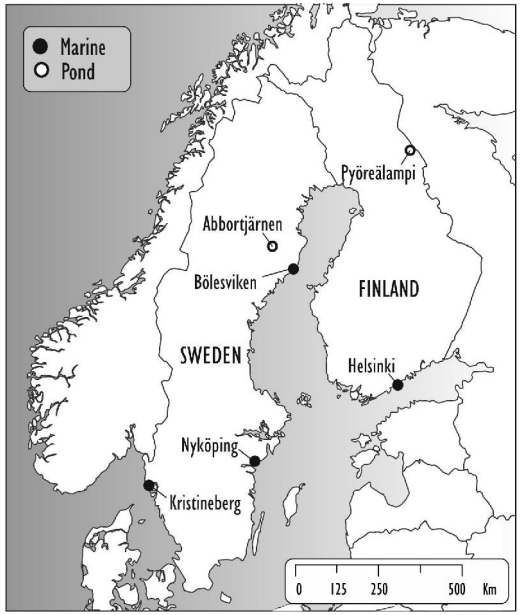
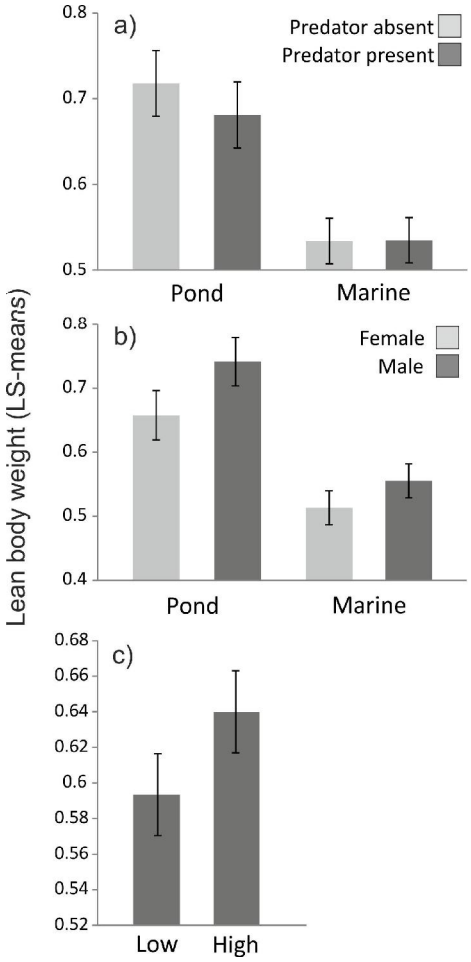


Figure 2. Least square means (± S.E) for the significant terms in the GLMM of lean body weight. a) Habitat × predation treatment interaction, b) Habitat × food treatment interaction, c) Sex effect.



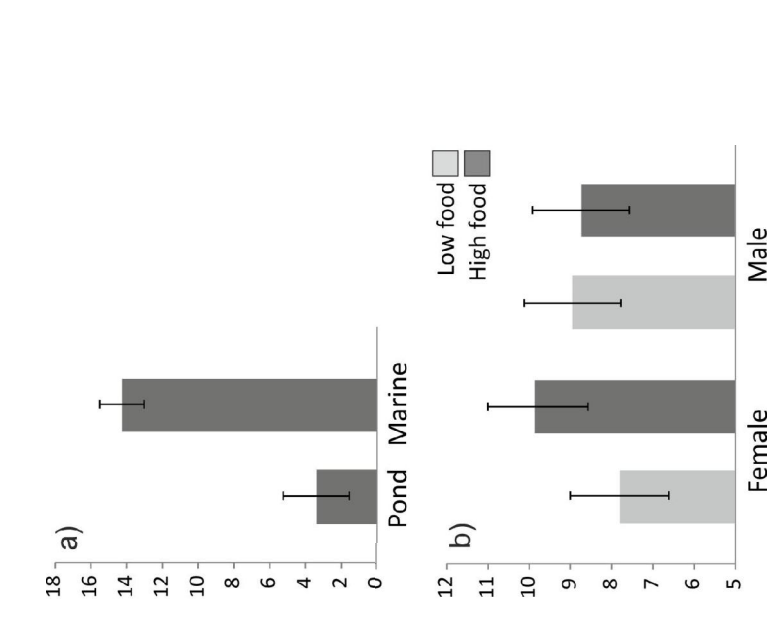


Figure 3. Least square means (± S.E) for the significant terms in the GLMM of fat body weight. a) Habitat effect, b) Sex × food treatment interaction.

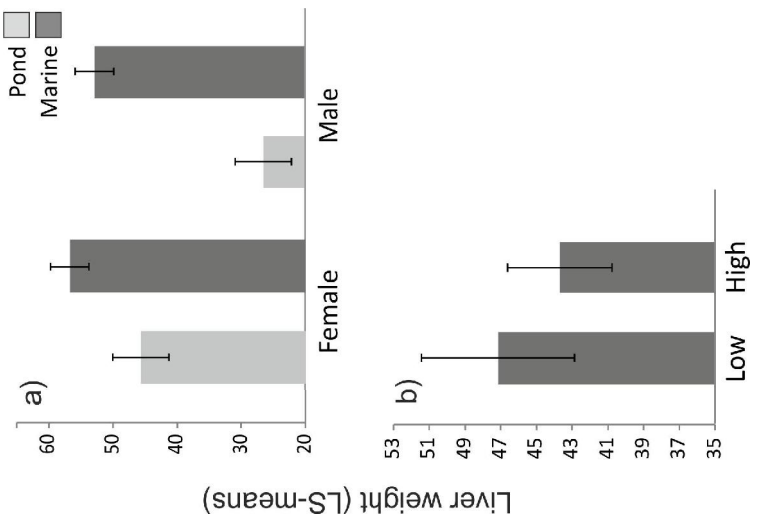


Figure 4. Least square means (± S.E) for the significant terms in the GLMM of liver weight. a) Habitat × sex interaction b) food treatment effects.

Local adaptation and phenotypic plasticity in the lateral line organs – an experiment

Kaisa Välimäki[1], Gábor Herczeg[1,*], Juha Merilä[1]

[1] *Ecological Genetics Research Unit, Department of Biosciences, PO Box 65, FI-00014 University Helsinki, Finland*

[*] *Present address: Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/C, H-1117, Hungary*

Correspondence to: KV

E-mail: kaisa.valimaki@helsinki.fi

Running head: Phenotypic plasticity in lateral line system.

Abstract

Sensory organs – including lateral-line organs in fish - are crucial for fitness, but little is known about their sensitivity to environmental influences during development. Here, we tested if perceived predation risk and food availability affected the development of the nine-spined stickleback (*Pungitius pungitius*) lateral line system (nine superficial and four canal neuromast traits) using replicated predation-adapted marine and competition-adapted pond populations subjected to a factorial common garden experiment with predation risk and food treatments. We found habitat-specific population divergence in four superficial neuromast and predation risk induced plasticity in several of the neuromast groups. However, complex interactions between habitat of origin, sex, predation risk and food treatments in two superficial neuromast traits complicated the interpretation of some the results. Nevertheless, the findings demonstrate that both genetic and plastic effects underlie variation in lateral line system, and that inducible effects are possible.

Key-words: Phenotypic plasticity, intraspecific variation, predation, competition, Lateral line

Introduction

Animals rely on sensory organs for information gathering to obtain food, mates and to avoid predation. Intraspecific variation in sensory capacity can therefore have large impact on the fitness of an individual. While some studies have shown intraspecific divergence in sensory system development and consistency (reviewed in Dangles et al., 2009), the role of adaptive phenotypic plasticity in this context remains largely unexplored (Dangles *et al.*, 2009). Studies that have shown phenotypic plasticity (i.e. production of different phenotypes from the same genotype as a response to same environmental cues; (West-Eberhard, 2003) in sensory organs have mainly been conducted on invertebrates. Examples include chemoreceptors in locusts (Opstad *et al.*, 2004), photoreceptor numbers in mantis shrimps (Cronin *et al.*, 2010) or compound eye structure in a butterfly (Merry *et al.*, 2011). Vertebrate studies have mainly concentrated on visual ability in fish (Kröger et al., 2001., Fuller et al., 2005; Chapman et al., 2010; Smith et al., 2011). Besides plasticity, another source for intraspecific variation is adaptation at genetic level as a response to differences in local environmental conditions (Kawecki & Ebert, 2004). Indeed, intraspecific variation in sensory systems has been found in many studies (Hansson et al., 1990; Löfstedt, 1990; Mcdonald & Hawryshyn, 1995; Cronin et al., 2002; Fuller et al., 2003; Spaethe, 2003; Boul et al., 2004; Siddiqi et al., 2004; Wark & Peichel, 2010). However, experimental studies conducted under common garden conditions allowing disentanglement of environmental and genetic causes of differentiation are almost completely lacking (but see: Fuller et al., 2005; Lavagnino et al., 2008; Merry et al., 2011; Trokovic et al., 2011). Furthermore, as far as we are aware of, possible differences in the degree of environmentally induced sensory plasticity between populations adapted to different environmental conditions have not been investigated as yet.

The mechanoreceptive lateral line system (hereafter LLS) of fish and aquatic amphibians detects small changes in water movement/pressure (Dijkgraaf, 1963; Bleckmann, 1993). LLS is used to recognize objects (Von Campenhausen *et al.*, 1981), prey and predators (Hoekstra & Janssen, 1985; Blaxter & Fuiman, 1990; McHenry *et al.*, 2009), being important for rheotaxis (Montgomery *et al.*, 1997), and for schooling behavior (Partridge & Pitcher, 1980; Faucher *et al.*, 2010). LLS is built up from neuromasts, which consist of small hair cells (cilia) within a gelatinous cupula (Bleckmann & Zelick, 2009). Superficial neuromasts are located on skin surface where they sense changes in water velocity on the close proximity of the fish (Kalmijn, 1987; Netten, 2006). The canal neuromasts are embedded in the skin in small fluid filled canals. Water acceleration near the body causes hydrodynamic pressure that moves the fluid within the canals, which canal neuromast can detect (Engelmann *et al.*, 2002). There is substantial interspecific variation in the number, position and size of both canal and superficial neuromasts (Coombs *et al.*, 1988; Webb, 1989), but only few intraspecific studies compared neuromast numbers among populations originating from different habitats (Michel et al., 2008; Wark & Peichel, 2010; Trokovic et al., 2011, 2012).

Nine-spined stickleback (*Pungitius pungitius*) offers an excellent model to study phenotypic plasticity and population variation in plasticity of the lateral line system. It is a small teleost fish that occurs in all kinds of freshwater systems and marine habitats throughout the northern hemisphere (Banarescu & Paepke, 2001). Previous studies have established that there are two distinct ecomorphs of the nine-spined stickleback in Fennoscandia showing adaptive divergence in morphology (Herczeg *et al.*, 2010), behaviour (Herczeg *et al.*, 2009b), life-history (Herczeg *et al.*, 2009a; c, 2012) and neuroanatomy (Gonda *et al.*, 2009, 2011).

The pond ecomorph occupies piscine-predation-free isolated ponds. In comparison to small marine ecomorph which lives in sympatry with various species of piscine predators, the pond ecomorph has diminished defensive armour, it is antisocial, aggressive and grows to giant sized adults. The selective pressures in pond and marine environments have also led to habitat-specific population differentiation in phenotypic plasticity between the ecomorphs in behavior, body size and relative size of different brain parts (Gonda *et al.*, 2011; Herczeg & Välimäki, 2011; Välimäki & Herczeg, 2012). Further, recent studies done in the same system of nine-spined stickleback have uncovered considerable population variation in the lateral line system (Trokovic *et al.*, 2011, 2012). Comparison of wild and common-garden reared nine-spined sticklebacks revealed that variation in neuromast numbers was much higher in wild populations, indicating environmentally induced variation and plasticity in neuromast numbers (Trokovic *et al.*, 2011).

The aim of the present study was to investigate experimentally the sources of intraspecific variation in neuromast numbers in the nine-spined stickleback lateral-line system. We reared nine-spined stickleback from predation-adapted marine and competition-adapted pond populations in a factorial common garden experiment with simultaneous manipulation of perceived predation risk and food supply. We hypothesised that (i) perceived predation risk should result in increased neuromast numbers, (ii) reduced food availability would constraint the strength of predation-induced response, and (iii) populations locally adapted to piscine predation would display a stronger plastic responses towards olfactory cues from predators as compared to individuals from populations locally adapted to a piscine-predator-free environment.

Material & Methods

Sampling and rearing

The parental fish were collected from two ponds (ABB, Abbortjärnen; PYÖ, Pyöreälampi) and from two coastal marine sites (HEL, Helsinki; KRI, Kristineberg) at the beginning of reproductive season in early summer 2009 (Fig. 1). The two ponds were small (surface area < 5 ha) and excluding a small number of recently introduced whitefish (*Coregonus lavaretus)* in Pyöreälampi, free of sympatric fish species. As whitefish are planktivorous, we expect them to be only competitors, and not predators to the nine-spined stickleback (Kahilainen *et al.*, 2004). Marine sampling sites are characterized with diverse fish fauna including several piscine predators.

Once the parental generation reached reproductive condition in the aquacultural facility of University of Helsinki, we produced full sib families via artificial fertilisation. 6-9 full sib families per population (PYÖ = 7, ABB = 6, HEL = 7, KRI = 9) were produced via artificial fertilisation. For fish fertilisation and husbandry we applied methods described in Välimäki et al. (2012). In short, freshly hatched fry were placed into individual 1.4 L containers in one of the four Allentown Zebrafish Rack Systems (Aquaneering Inc., San Diego, CA, USA). Four treatment combinations used, and the aim was to have equal population and family representation in each treatment. Fish were kept in constant +12°C and photoperiod was set to 14 light:10 dark. Fish were initially fed with live brine shrimp nauplii (*Artemia* sp.) and gradually changed to feed frozen Chironomid larvae after 80 days of rearing.

Treatments

Fish were divided into four treatments in a factorial experiment with predation risk and food treatments, each with two levels. For the predation treatment we connected an extra 150 L plastic tank into each rack. Water - first filtered by biological and mechanical filters - circulated through these tanks and then to the individual containers within the rack. Two perches (*Perca fluviatilis*) were placed into two of the extra containers to provide the olfactory cues of predators to the experimental fish. The two remaining extra tanks were controls without predators. For the food treatment, fish within families, predation treatments and racks were divided randomly into high and low food level groups, being fed *in excess* two times per day or once per two days, respectively. We note that even though perch is abundant predator in Fennoscandian freshwaters and in the Baltic Sea, it does not occur in Atlantic side of the Swedish coast from where Kristineberg individuals originate. However, in previous studies (Herczeg & Välimäki, 2011; Välimäki & Herczeg, 2012) we have shown that Kristineberg individuals exhibit both behavioural and growth–related responses to the perch–predation risk treatment, suggesting that they are able to recognize perch as a predator.

Measurements

After 34 weeks of rearing, a subsample of all individuals was chosen for the neuromast counting. We used 171 fish (PYÖ = 50, ABB = 37, HEL = 41 and KRI = 43). For detection of neuromasts, a common procedure for staining with vital fluorescent dye DASPEI (diluted to a working concentration of 0.025% with fresh fish-tank water) was used (Balak *et al.*, 1990). DASPEI is a voltage-sensitive dye that specifically labels the hair cells of the lateral line neuromasts and the nasal epithelium of the fish. First fish were allowed to swim freely in the staining solution for 15 min, after which they were anesthetized in 0.016% MS-222 (tricaine methylsulfonate). Neuromasts were observed and counted under a Leica fluorescence dissecting microscope with a FITC filter set (Leica Microsystems Inc., Bannockburn, IL, USA). We used the following lateral lines (depicted in Fig. 2; canal neuromasts are indicated with –c suffix): post-otic (POt-c), pre-opercular (Pr-c), supraorbital (SO-c), anterior trunk (ATr-c), upper mandibular preopercular (MPrU), mandibular (M), pre-opercular (Pr), infraorbital (IO), otic (Ot), post-orbital (PO), dorsal head (DH), operculum (OP) and anterior trunk (ATr). Sex was identified by dissection and visual inspection of the gonads.

Statistical analyses

We first explored the data and based on the variation and distribution of neuromasts in individual lateral lines we excluded three canal neuromasts due to lack of sufficient variation for analyses (POt-c, Pr-c, SO-c; Table 1).

The remaining 10 neuromast groups (M, MPrU, Pr, IO, OT, PO, DH, OP, ATr, ATr-c) were analyzed using Generalized Linear Mixed Models (GLMMs). As the traits represent count data, parametric models couldn't be used. In a recent simulation study, O'Hara & Kotze (2010) showed that linear models with Poisson or negative binomial distributions perform better than the traditional Log_{10} transformation. Hence, GLMMs with Poisson distribution and log link function were used. Due to the large number of nonindependent statistical tests, some kind of correction method was necessary. Because of the criticism on

Bonferroni-type of corrections (Benjamini & Hochberg, 1995; Nakagawa, 2004; Narum, 2006), we applied the False Discovery Rate approach of Benjamini & Hochberg (1995). It was based on the pool of the significant effects from all final models (model selection method is outlined below). Habitat- and population-based models (see below) were considered separately.

We were interested in the environmental effects on neuromast numbers both on evolutionary (habitat and population effects), and ontogenetic (treatment effects) scales together with possible sex effects and the possible interactions. Hence the traits were analyzed separately first on habitat level using habitat (marine *vs.* pond), sex, food and predation treatments as fixed factors and population nested with habitat as random factor and then on population level with population, sex, food and predation treatments as fixed factors. The initial full models consisted of all main effects and their interactions up to the four-way level. We applied backward stepwise model selection based on the $P < 0.05$ criterion. This was done by starting with the highest level interaction progressing towards main effects in a way that any effect included in a significant higher level interaction was not removed at lower interactions and main effects. This approach is considered conservative as compared to other model selection procedures (Murtaugh, 2009).

The GLMM on ATr-C was dismissed because we found that the pond populations almost invariantly lacked these neuromasts. Because marine populations showed high variation in ATr-c numbers, we ran a restricted GLMM on this trait, comparing the two marine populations, sexes, food- and predation treatments. All statistical analyses were conducted with SAS 9.2 (SAS Institute Inc., Cary, NC, USA) package.

Results

In general, the lateral line system was highly variable (Table 1). Both habitat (ATr & OP, Table 2, Figs. 3, 4.) and population (M, MPrU, Pr, IO, Ot, PO, DH, OP, ATr) specific divergence in neuromast numbers were observed (Table S2, Figs. S1, S2, S3;). There were also complex interactions between habitat, predation treatment and sex in two (ATr & OP) out of the 13 studied traits (Table 1, Figs. 3, 4). In addition, there were also simpler predation treatment effects on population level on two traits (DH & OT), in which perceived predation risk increased neuromast numbers (Table S2, Fig. S2). Because the specific functions of the different neuromast groups are unknown, we describe the complex patterns on habitat level in detail below to highlight the potential factors affecting variation in neuromast numbers, while the simpler population level results are only shown in electronic support material.

In OP, there was a significant habitat × predation interaction (Table 2). Marine populations had higher neuromast counts than pond populations in general. Further, pond individuals under perceived predation risk had more OP neuromasts than in absence of perceived predation risk, while this effect was absent in marine populations (Fig. 3).

In ATr, we found a significant habitat × sex × food × predation interaction (Table 2). As four-way interactions are notoriously hard to interpret, we ran separate GLMMs for the sexes to aid the interpretation of patterns. Again habitat, food and predation treatments and their interactions were used (model selection similar to the other models; Table S1). In females, we found significant habitat × food × predation interaction, while in males there were significant habitat × predation and habitat × food interactions (Table S3). Visual inspection of the complex patterns (Fig. 4) identified the following patterns. There was a clear habitat effect: marine fish had more neuromasts than pond fish. In females, only marine fish responded to the treatments: in the low food treatment, neuromast numbers tended to increase under perceived predation risk, while in the high food treatment the opposite was true (Fig. 4). In males, pond fish responded to predation treatment with increased number of neuromasts, while marine males in low food treatment had lower number of neuromasts as a response to predation treatment. Pond males developed more neuromasts in high food treatment as compared to low food treatment (Fig. 4).

ATr-c showed very strong habitat specific divergence in neuromast numbers. Almost all individuals originating from pond populations lacked neuromast in this area while the variation was from 1 to 10 in marine populations. However, population-level analysis on marine populations did not reveal any significant explanatory effects (Table S4).

Discussion

Patterns of environmentally induced variation in sensory organs are currently largely unexplored, especially in vertebrates (Fuller *et al.*, 2005; Dangles *et al.*, 2009). Further, our knowledge about intraspecific lateral line system variability is very limited, and mostly based on simple population comparisons (Michel *et al.*, 2008; Wark & Peichel, 2010; Trokovic *et al.*, 2011, 2012). The results of the present study demonstrate considerable intraspecific variability in neuromast numbers in nine-spined stickleback, and provide evidence that neuromast numbers exhibit phenotypic plasticity. Furthermore, the results suggest local adaptations, and also show how evolutionary history (habitat of origin) or sex can affect the expression of phenotypic plasticity. Although we are unable to draw firm causative conclusions from the present study, it does constitute an important pioneering step towards understanding the microevolutionary and plastic sources of variation in the development of sensory organs, and those of lateral line system in particular. In what follows, we will outline and discuss the most salient findings of this study.

Plasticity in LLS

It has been suggested that plasticity in sensory organs is likely to be substantial, but further studies have called for to verify the generality of this hypothesis (Dangles *et al.*, 2009). One of the main findings here was that predation treatment increased neuromast numbers in certain lateral line groups suggesting that nine-spined sticklebacks can adjust neuromast numbers as a response to threat of predation. There is evidence that higher number and higher density of neuromasts can increase the resolution of lateral line system (Coombs *et al.*, 1988; Yoshizawa *et al.*, 2010; Yoshizawa & Jeffery, 2011). By inference, more sensitive sensory system can facilitate predator avoidance or recognition in nine-spined stickleback. In general, predator-induced plasticity is common in aquatic organisms and has been shown to affect wide variety of fitness related traits such as behaviours (Relyea, 2002; Biro *et al.*, 2005; Steiner, 2007), morphology (Stemberger & Gilbert, 1984; Tollrian & Harwell, 1999; Teplitsky *et al.*, 2005; Andersson *et al.*, 2006; Walker & McCormick, 2009; Abate *et al.*, 2010) and even neuroanatomical traits likes size of different brain parts (Gonda *et al.*, 2010,2011). To our knowledge, predator induced plasticity in sensory organs has not been demonstrated before, but for instance, the development of the sensory cercal structures in crickets appear to be related to variation predation pressure (Dangles *et al.*, 2006). Considering that lateral

line system is an important sensory organ in predation avoidance (Blaxter & Fuiman, 1990; McHenry *et al.*, 2009), the ability to plastically react to perceived threat of predation indicates possible existence of adaptive plasticity in neuromast numbers.

We also found that in case of neuromasts in ATr-group, food treatment affected the expression of predator-induced plasticity. However, due to interactions with other factors, the effects were difficult to interpret. In pond males high food treatment increased the number of neuromasts, but in marine females the high food treatment had the opposite effect. It is known that energetic state of an individual can affect the expression of predator-induced plasticity (Noonburg & Nisbet, 2005; Teplitsky *et al.*, 2005; Chivers *et al.*, 2007; Borcherding & Magnhagen, 2008). Usually individuals in better condition can invest more on defensive traits, though opposite patterns can also be found (Pauwels *et al.*, 2010). Also other sensory organs, such as compound eye structure has been shown to respond plastically to food limitation: Orange Sulphur butterflies subject to low food treatment developed proportionally larger eyes, indicating increased investment on vision under food stress (Merry *et al.*, 2011). At any rate, we found evidence that olfactory cues from a predator – even without visual cues or physical contact – can alter the potential prey's lateral line development.

Local adaptation in phenotypic plasticity

It is not entirely clear how do local adaptation and phenotypic plasticity interact, and this issue has spurred up a vigorous debate (DeWitt & Scheiner, 2004; de Jong, 2005; Pigliucci *et al.*, 2006; Crispo, 2008; Fusco & Minelli, 2010; Pfennig *et al.*, 2010). Plasticity can be seen as a process that either impedes or facilitates adaptive divergence and speciation, or as a quantitative trait that is itself under selection. It seems to be clear that the degree of plasticity can change in the course of evolution depending on the scale of environmental and temporal variation (Gomulkiewicz & Kirkpatrick, 1992; Moran, 1992; Sultan & Spencer, 2002; Ernande & Dieckmann, 2004; Baythavong, 2011). When environmental variation is low, a local population might lose its environmental sensitivity through genetic assimilation, leading to the fixation of the optimal phenotype (Waddington, 1953; Crispo, 2007). On the other hand, when environmental variation is high and predictable, phenotypic plasticity might be favoured over fixed genotypes. From a sensory system point-of–view, studies of population or habitat specific variation in plasticity are lacking, though the need for them has been acknowledged (Dangles *et al.*, 2009). We found that perceived predation risk affected two lateral line groups in a habitat and population-dependent way. In the OP neuromast group pond sticklebacks developed more neuromasts in the presence than in the absence of predator, while marine sticklebacks had invariantly more neuromasts than their pond conspecifics. These results suggest that in marine environments selection is stronger for higher neuromast number in OP, and that the neuromast number of this neuromast group became fixed, whereas in pond environments where neuromast number was smaller, plasticity is present. As larger number of neuromasts can increase the sensitivity to sensory stimulus (Coombs & Montgomery, 1999; Yoshizawa *et al.*, 2010; Yoshizawa & Jeffery, 2011) the patterns we observe in neuromast numbers on OP neuromast group can be indicative of the important role that it has in predator recognition. Even though the pond populations have been adapted to the absence of predators, and showed a decrease in neuromast numbers on this neuromast group, they displayed plastic response to the predator presence. This pattern is intriguing especially considering that marine fish might represent the ancestral form. However similar pattern was found in olfactory bulb size of nine-spined stickleback brains (Gonda *et al.*, 2011). The complex patterns found from ATr similarly suggest that the evolutionary history affects the development of lateral line system, though the overall picture is not as clear as in the case of OP.

Habitat-dependent population divergence of plasticity in nine-spined stickleback has been shown in growth (Välimäki & Herczeg, 2012), behaviour (Herczeg & Välimäki, 2011) and brain architecture (Gonda *et al.*, 2011), but not in general morphology (*viz.* body shape and armour; (Välimäki *et al.*, 2012). In this study, some parts of the lateral line system showed habitat dependent signs of predator induced plasticity, while others did not. The habitat-dependence of phenotypic plasticity patterns derived from replicated populations suggests that natural selection has shaped expression of plasticity. While the low number of replicate populations in our study cautions against broad generalizations, the results suggests that like many other traits, also some of the lateral-line neuromast groups express habitat-dependent population divergence in their capacity for plasticity.

Local adaptation

The separation of genetically based local adaptation from environmentally induced phenotypic plasticity using common garden or similar approaches is essential to understand the evolutionary significance of trait divergence (Conover & Schultz, 1995; Gotthard & Nylin, 1995; Kawecki & Ebert, 2004). Studies where the environmental and genetic components can be disentangled have been seldom done on sensory organs (Fuller *et al.*, 2005; Dangles *et al.*, 2009; Merry *et al.*, 2011), though number of studies have demonstrated intraspecific phenotypic differences in different sensory organs (Hansson *et al.*, 1990; McClelland *et al.*, 1998; Chittka *et al.*, 2004; Opstad *et al.*, 2004; Siddiqi *et al.*, 2004). Here we raised fish individually from egg stage to near maturity in laboratory environment where we could control environmental influences on lateral line system development. Thus, the observed differences in lateral line numbers are either caused by the treatments (reflecting phenotypic plasticity) or in case we observe habitat (or population) specific effects, they are likely to have genetic basis- a perquisite for postulating adaptive explanations for the observed divergence. Of course, as our results are based on F1-generation fish, we cannot fully exclude maternal or cross-generational influences on phenotypes (Lynch & Walsh, 1998). This is a common problem with studies of species where rearing several generations in the lab is a logistical challenge.

We found that neuromast number was smaller in pond than in marine populations in three neuromast groups (OP, ATr, ATr-c). In the case of ATr-c, the divergence was so strong that most of the pond individuals did actually lack this lateral line. The repeated, independent occurrence of the same phenotype in environmentally similar populations suggests that natural selection is the causal agent of the observed differences (Clarke, 1975; Endler, 1986; McGuigan *et al.*, 2005). Pond environments are simpler than marine environments in many aspects. The ponds we sampled are small, isolated water bodies that lack other fish species than nine-spined sticklebacks, have negligible aquatic vegetation, currents, and physical complexity. Therefore, the demand for a high-tuned and potentially costly sensory system is likely to be lower in ponds as compared to marine sites. However, in many occasions the patterns found at population level did not match those observed at habitat level suggesting that there are other factors than predation risk that shape the neuromast numbers. Further

studies aiming to link variation in neuromast numbers to behaviours and functional differences should shed more light on possible explanations behind this heterogeneity.

Conclusions

In conclusion, we found that the nine-spined stickleback lateral line system is a highly variable sensory organ subject to both plastic and genetic effects. While some parts of it are environmentally inducible both on the ontogenetic and evolutionary time scales, others show minimal variation. We found support for the roles of both phenotypic plasticity and local adaptation, as well as their interaction in shaping the patterns of neuromast population differentiation in the wild. Hence, given the high intraspecifc variability both within and among populations, lateral line system provides a good model system for studies seeking to understand microevolution and phenotypic plasticity in sensory organs. This is particularly in view that neuromasts can be easily counted with minimal error, and there exists advanced methods to study their function. Future studies should address how this important organ evolves as a response to local environmental variation, and how both genetic and environmentally induced components of phenotypic variation in lateral line phenotypes influence individual fitness.

Acknowledgements

Jacquelin DeFaveri, Göran Englund, Jenni Hämäläinen, Lotta Kvarnemo, Tuomas Leinonen, Pia Rännänen and Pirkko Siikamäki helped to obtain the samples. Mirva Turtiainen, Linda Uoti, Maarit Haataja and Marika Lilja helped with fish maintenance. Marina Bogomolov consulted us with the false discovery rate–correction. Our work was supported by Academy of Finland (GH, # 128716; JM, #134728 & #129662), Finnish Graduate School in Population Genetics, Eemil Aaltonen Foundation and Georg and Ella Ehrnrooth Foundation and Kordelin Foundation (KV). The experiment was conducted under the license of the Finnish National Animal Experiment Board (Licence number STH223A).

Literature

Abate, M.E., Eng, A.G. & Kaufman, L. 2010. Alarm cue induces an antipredator morphological defense in juvenile Nicaragua cichlids *Hypsophrys nicaraguensis*. *Current Zoology* **56**: 36–42.

Andersson, J., Johansson, F. & Söderlund, T. 2006. Interactions between predator- and diet-induced phenotypic changes in body shape of crucian carp. *Proceedings of the Royal Society B* **273**: 431–437.

Balak, K.J., Corwin, J.T. & Jones, J.E. 1990. Regenerated hair cells can originate from supporting cell progeny: evidence from phototoxicity and laser ablation experiments in the lateral line system. *The Journal of Neuroscience* **10**: 2502–2512.

Banarescu, P.M. & Paepke, H.J. 2001. *The Freshwater Fishes of Europe, Vol 5/III.* AULA-Verlag, Wiebelsheim.

Baythavong, B.S. 2011. Linking the spatial scale of environmental variation and the evolution of phenotypic plasticity: selection favors adaptive plasticity in fine-grained environments. *American Naturalist* **178**: 75–87.

Benjamini, Y. & Hochberg, Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, Series B* **57**: 289–300.

Biro, P.A., Post, J.R. & Abrahams, M. V. 2005. Ontogeny of energy allocation reveals selective pressure promoting risk-taking behaviour in young fish cohorts. *Proceedings of the Royal Society B* **272**: 1443–1448.

Blaxter, J.H.S. & Fuiman, L.A. 1990. The role of the sensory systems of herring larvae in evading predatory fishes. *Journal of the Marine Biological Association of the United Kingdom* **70**: 413–427.

Bleckmann, H. 1993. Role of the lateral line in fish behaviour. In: *Behaviour of Teleost Fishes* (T.J. Pitcher, ed), pp. 201–246. Chapman & Hall, London.

Bleckmann, H. & Zelick, R. 2009. Lateral line system of fish. *Integrative Zoology* **4**: 13–25.

Borcherding, J. & Magnhagen, C. 2008. Food abundance affects both morphology and behaviour of juvenile perch. *Ecology of Freshwater Fish* **17**: 207–218.

Boul, K.E., Ryan, M.J. & Ryan, J. 2004. Population variation of complex advertisement calls in Physalaemus petersi and comparative laryngeal morphology. *Copeia* **2004**: 624–631.

Chapman, B.B., Morrell, L.J., Tosh, C.R. & Krause, J. 2010. Behavioural consequences of sensory plasticity in guppies. *Proceedings of the Royal Society B* **277**: 1395–1401.

Chittka, L., Ings, T.C. & Raine, N.E. 2004. Chance and adaptation in the evolution of island bumblebee behaviour. *Population Ecology* **46**: 243–251.

Chivers, D.P., Zhao, X., Brown, G.E., Marchant, T.A. & Ferrari, M.C.O. 2007. Predator-induced changes in morphology of a prey fish: the effects of food level and temporal frequency of predation risk. *Evolutionary Ecology* **22**: 561–574.

Clarke, B. 1975. The contribution of ecological genetics to evolutionary theory: detecting the direct effects of natural selection on particular polymorphic loci. *Genetics* **79**: 101–113.

Conover, D.O. & Schultz, E.T. 1995. Phenotypic similarity and the evolutionary significance of countergradient variation. *Trends in Ecology and Evolution* **10**: 248–252.

Coombs, S., Janssen, J. & Webb, J.F. 1988. Diversity of lateral line systems, evolutionary and functional considerations. In: *Sensory Biology of Aquatic Animals* (J. Atema, R. R. Fay, A. N. Popper, & W. N. Tavolga, eds), pp. 553–593. Springer-Verlag, Heidelberg.

Coombs, S. & Montgomery, J.C. 1999. The enigmatic Lateral Line System. In: *Comparative Hearing: Fish and Amphibians* (Richard R. Fay & Arthur N. Popper, eds), pp. 319–362. Springer New York, New York, NY.

Crispo, E. 2008. Modifying effects of phenotypic plasticity on interactions among natural selection, adaptation and gene flow. *Journal of Evolutionary Biology* **21**: 1460–1469.

Crispo, E. 2007. The Baldwin effect and genetic assimilation: revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. *Evolution* **61**: 2469–2479.

Cronin, T.W., Caldwell, R.L. & Erdmann, M. V. 2002. Tuning of photoreceptor function in three mantis shrimp species that inhabit a range of depths. I. Visual pigments. *Journal of Comparative Physiology A* **188**: 179–186.

Cronin, T.W., Caldwell, R.L. & Marshall, J. 2010. Tunable colour vision in a mantis shrimp. *Nature* **411**: 2000–2001.

Dangles, O., Irschick, D., Chittka, L. & Casas, J. 2009. Variability in sensory ecology: expanding the bridge between physiology and evolutionary biology. *The Quarterly Review of Biology* **84**: 51–74.

Dangles, O., Pierre, D., Magal, C., Vannier, F. & Casas, J. 2006. Ontogeny of air-motion sensing in cricket. *Journal of Experimental Biology* **209**: 4363–70.

De Jong, G. 2005. Evolution of phenotypic plasticity: patterns of plasticity and the emergence of ecotypes. *The New Phytologist* **166**: 101–117.

DeWitt, T.J. & Scheiner, S.M. 2004. *Phenotypic Plasticity: Functional and Conceptual Approaches*. New York: Oxford University Press.

Dijkgraaf, S. 1963. The functioning and significance of the lateral line organs. *Biological Reviews* **38**: 051–105.

Endler, J.A. 1986. *Natural Selection in the Wild*. Princeton University Press, Princeton.

Engelmann, J., Hanke, W. & Bleckmann, H. 2002. Lateral line reception in still- and running water. *Journal of Comparative Physiology A* **188**: 513–526.

Ernande, B. & Dieckmann, U. 2004. The evolution of phenotypic plasticity in spatially structured environments: implications of intraspecific competition, plasticity costs and environmental characteristics. *Journal of Evolutionary Biology* **17**: 613–628.

Faucher, K., Parmentier, E., Becco, C., Vandewalle, N. & Vandewalle, P. 2010. Fish lateral system is required for accurate control of shoaling behaviour. *Animal Behaviour* **79**: 679–687. Elsevier Ltd.

Fuller, R.C., Carleton, K L, Fadool, J.M., Spady, T.C. & Travis, J. 2005. Genetic and environmental variation in the visual properties of bluefin killifish, *Lucania goodei*. *Journal of Evolutionary Biology* **18**: 516–523.

Fuller, R.C., Fleishman, L.J., Leal, M., Travis, J. & Loew, E. 2003. Intraspecific variation in retinal cone distribution in the bluefin killifish, *Lucania goodei*. *Journal of Comparative Physiology A* **189**: 609–616.

Fusco, G. & Minelli, A. 2010. Phenotypic plasticity in development and evolution: facts and concepts. *Philosophical Transactions of the Royal Society B* **365**: 547–556.

Gomulkiewicz, R. & Kirkpatrick, M. 1992. Quantitative genetics and the evolution of reaction norms. *Evolution* **46**: 390–411.

Gonda, A., Herczeg, G. & Merilä, J. 2009. Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? *Journal of Evolutionary Biology* **22**: 1721–1726.

Gonda, A., Herczeg, G. & Merilä, J. 2011. Population variation in brain size of nine-spined sticklebacks (*Pungitius pungitius*)--local adaptation or environmentally induced variation? *BMC Evolutionary Biology* **11**: 75.

Gonda, A., Trokovic, N., Herczeg, G., Laurila, A. & Merilä, J. 2010. Predation- and competition-mediated brain plasticity in Rana temporaria tadpoles. *Journal of Evolutionary Biology* **23**: 2300–2308.

Gonda, A., Välimäki, K., Herczeg, G. & Merilä, J. 2011. Brain development and predation: plastic responses depend on evolutionary history. *Biology Letters* **8**: 249–252.

Gotthard, K. & Nylin, S. 1995. Adaptive plasticity and plasticity as an adaptation?: a selective review of plasticity in animal morphology and life history. *Oikos* **74**: 3–17.

Hansson, B.S., Tóth, M., Löfstedt, C., Szöcs, G., Subchev, M. & Löfqvist, J. 1990. Pheromone variation among eastern european and a western asian population of turnip moth *Agrotis segetum*. *Journal of Chemical Ecology* **16**.

Herczeg, G., Gonda, A., Kuparinen, A. & Merilä, J. 2012. Contrasting growth strategies of pond versus marine populations of nine-spined stickleback (*Pungitius pungitius*): a combined effect of predation and competition? *Evolutionary Ecology* **26**: 109–122.

Herczeg, G., Gonda, A. & Merilä, J. 2009a. Evolution of gigantism in nine-spined sticklebacks. *Evolution* **63**: 3190–3200.

Herczeg, G., Gonda, A. & Merilä, J. 2009b. Predation mediated population divergence in complex behaviour of nine-spined stickleback (*Pungitius pungitius*). *Journal of Evolutionary Biology* **22**: 544–552.

Herczeg, G., Gonda, A. & Merilä, J. 2009c. The social cost of shoaling covaries with predation risk in nine-spined stickleback, *Pungitius pungitius*, populations. *Animal Behaviour* **77**: 575–580.

Herczeg, G., Turtiainen, M. & Merilä, J. 2010. Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): *Biological Journal of the Linnean Society* **101**: 403–416.

Herczeg, G. & Välimäki, K. 2011. Intraspecific variation in behaviour: effects of evolutionary history, ontogenic experience and sex. *Journal of Evolutionary Biology* **24**: 2434–2444.

Hoekstra, D. & Janssen, J. 1985. Non-visual feeding behavior of the mottled sculpin, *Cottus bairdi*, in Lake Michigan. *Environmental Biology of Fishes* **12**: 111–117.

Kahilainen, K., Malinen, T., Tuomaala, A. & Lehtonen, H. 2004. Diel and seasonal habitat and food segregation of three sympatric *Coregonus lavaretus* forms in a subarctic lake. *Journal of Fish Biology* 418–434.

Kalmijn, A.J. 1987. Hydrodynamic and acoustic field detection. In: *Sensory Biology of Aquatic Animals* (J. Atema, R.R. Fay, A.N. Popper, & W. N. Tavolga, eds), pp. 83–130. Springer-Verlag, Heidelberg.

Kawecki, T.J. & Ebert, D. 2004. Conceptual issues in local adaptation. *Ecology Letters* **7**: 1225–1241.

Kröger, R.H., Campbell, M.C. & Fernald, R.D. 2001. The development of the crystalline lens is sensitive to visual input in the African cichlid fish, *Haplochromis burtoni*. *Vision research* **41**: 549–559.

Lavagnino, N.J., Anholt, R.R.H. & Fanara, J.J. 2008. Variation in genetic architecture of olfactory behaviour among wild-derived populations of Drosophila melanogaster. *Journal of Evolutionary Biology* **21**: 988–996.

Lynch, M. & Walsh, J.B. 1998. *Genetics and Analysis of Quantitative Traits*. Sinauer Associates, Inc., Sunderland, MA.

Löfstedt, C. 1990. Population variation and genetic control of pheromone communication systems in moths. *Entomologia Experimentalis et Applicata* **54**: 199–218.

McClelland, B.E., Wilczynski, W. & Ryan, M.J. 1998. Intraspecific variation in laryngeal and ear morphology in male cricket frogs (*Acris crepitans*). *Biological Journal of the Linnean Society* **63**: 51–67.

McDonald, G. & Hawryshyn, W. 1995. Intraspecific variation of spectral sensitivity in threespine stickleback (*Gasterosteus aculeatus*) from different photic regimes. *Journal of Comparative Physiology A* **176**: 255–260.

McGuigan, K., Chenoweth, S.F. & Blows, M.W. 2005. Phenotypic divergence along lines of genetic variance. *American Naturalist* **165**: 32–43.

McHenry, M.J., Feitl, K.E., Strother, J.A. & Van Trump, W.J. 2009. Larval zebrafish rapidly sense the water flow of a predator's strike. *Biology Letters* **5**: 477–479.

Merry, J.W., Kemp, D.J. & Rutowski, R.L. 2011. Variation in compound eye structure: effects of diet and family. *Evolution* **65**: 2098–2110.

Michel, C., Hicks, B.J., Stölting, K.N., Clarke, A.C., Stevens, M.I., Tana, R., *et al.* 2008. Distinct migratory and non-migratory ecotypes of an endemic New Zealand eleotrid (*Gobiomorphus cotidianus*) - implications for incipient speciation in island freshwater fish species. *BMC Evolutionary Biology* **8**: 49.

Montgomery, J.C., Baker, C.F. & Carton, A.G. 1997. The lateral line can mediate rheotaxis in fish. *Nature* **389**: 960–963.

Moran, N. 1992. The evolutionary maintenance of alternative phenotypes. *American Naturalist* **139**: 971–989.

Murtaugh, P.A. 2009. Performance of several variable-selection methods applied to real ecological data. *Ecology Letters* **12**: 1061–1068.

Nakagawa, S. 2004. A farewell to Bonferroni: the problems of low statistical power and publication bias. *Behavioral Ecology* **15**: 1044–1045.

Narum, S.R. 2006. Beyond Bonferroni: Less conservative analyses for conservation genetics. *Conservation Genetics* **7**: 783–787.

Netten, V.S.M. 2006. Hydrodynamic detection by cupulae in a lateral line canal: functional relations between physics and physiology. *Biological Cybernetics* **94**: 67–85.

Noonburg, E.G. & Nisbet, R.M. 2005. Behavioural and physiological responses to food availability and predation risk. *Evolutionary Ecology* **7**: 89–104.

O'Hara, R.B. & Kotze, D.J. 2010. Do not log-transform count data. *Methods in Ecology and Evolution* **1**: 118–122.

Opstad, R., Rogers, S.M., Behmer, S.T. & Simpson, S.J. 2004. Behavioural correlates of phenotypic plasticity in mouthpart chemoreceptor numbers in locusts. *Journal of Insect Physiology* **50**: 725–736.

Partridge, B.L. & Pitcher, T.J. 1980. The sensory basis of fish schools: relative roles of lateral line and vision. *Journal of Comparative Physiology A* **135**: 315–325.

Pauwels, K., Stoks, R. & De Meester, L. 2010. Enhanced anti-predator defence in the presence of food stress in the water flea *Daphnia magna*. *Functional Ecology* **24**: 322–329.

Pfennig, D.W., Wund, M.A., Snell-Rood, E.C., Cruickshank, T., Schlichting, C.D. & Moczek, A.P. 2010. Phenotypic plasticity's impacts on diversification and speciation. *Trends in Ecology and Evolution* **25**: 459–467.

Pigliucci, M., Murren, C.J. & Schlichting, C.D. 2006. Phenotypic plasticity and evolution by genetic assimilation. *Journal of Experimental Biology* **209**: 2362–2367.

Relyea, R.A. 2002. Local population differences in phenotypic plasticity: predator-induced changes in wood frog tadpoles. *Ecological Monographs* **72**: 77–93.

Siddiqi, A., Cronin, T.W., Loew, E.R., Vorobyev, M. & Summers, K. 2004. Interspecific and intraspecific views of color signals in the strawberry poison frog *Dendrobates pumilio*. *Journal of Experimental Biology* **207**: 2471–2485.

Smith, A.R., D'Annunzio, L., Smith, A.E., Sharma, A., Hofmann, C.M., Marshall, N.J., *et al.* 2011. Intraspecific cone opsin expression variation in the cichlids of Lake Malawi. *Molecular Ecology* **20**: 299–310.

Spaethe, J. 2003. Interindividual variation of eye optics and single object resolution in bumblebees. *Journal of Experimental Biology* **206**: 3447–3453.

Steiner, U.K. 2007. Investment in defense and cost of predator-induced defense along a resource gradient. *Oecologia* **152**: 201–210.

Stemberger, R.S. & Gilbert, J.J. 1984. Spine development in the rotifer *Keratella cochlearis*: induction by cyclopoid copepods and *Asplanchna*. *Freshwater Biology* **14**: 639–647.

Sultan, S.E. & Spencer, H.G. 2002. Metapopulation structure favors plasticity over local adaptation. *American Naturalist* **160**: 271–283.

Teplitsky, C., Plénet, S. & Joly, P. 2005. Costs and limits of dosage response to predation risk: to what extent can tadpoles invest in anti-predator morphology? *Oecologia* **145**: 364–370.

Tollrian, R. & Harwell, C. 1999. *The Ecology and Evolution of Inducible Defenses*. Princeton University Press, Princeton.

Trokovic, N., Herczeg, G., Ab Ghani, N.I. & Shikano, T. 2012. High levels of fluctuating asymmetry in isolated stickleback populations. *BMC Evolutionary Biology* **12**: 115

Trokovic, N., Herczeg, G., Scott McCairns, R.J., Ab Ghani, N.I. & Merilä, J. 2011. Intraspecific divergence in the lateral line system in the nine-spined stickleback (*Pungitius pungitius*). *Journal of Evolutionary Biology* **24**: 1546–1558.

Waddington, C.. 1953. The "'Baldwin effect,'" "'genetic assimilation'" and "'homeostasis'". *Evolution* **7**: 386–387.

Walker, S.P.W. & McCormick, M.I. 2009. Sexual selection explains sex-specific growth plasticity and positive allometry for sexual size dimorphism in a reef fish. *Proceedings of the Royal Society B* **276**: 3335–43.

Wark, A.R. & Peichel, C.L. 2010. Lateral line diversity among ecologically divergent threespine stickleback populations. *Journal of Experimental Biology* **213**: 108–117.

Webb, J.F. 1989. Gross morphology and evolution of the mechanoreceptive lateral-line system in teleost fishes. *Brain, Behavior and Evolution* **33**: 34–53.

West-Eberhard, M.J. 2003. *Developmental Plasticity and Evolution*. Oxford University Press.

Von Campenhausen, C., Riess, I. & Weissert, R. 1981. Detection of stationary objects by the blind cave fish *Anoptichthys jordani* (Characidae). *Journal of Comparative Physiology A* 369–374.

Välimäki, K. & Herczeg, G. 2012. Ontogenetic and evolutionary effects of predation and competition on nine-spined stickleback (*Pungitius pungitius*) body size. *Journal of Animal Ecology* **81**: 859–867.

Välimäki, K., Herczeg, G. & Merilä, J. 2012. Morphological antipredator defences in the nine-spined stickleback: constitutive, induced or both? *Biological Journal of the Linnean Society* in press.

Yoshizawa, M., Goricki, S., Soares, D. & Jeffery, W.R. 2010. Evolution of a behavioral shift mediated by superficial neuromasts helps cavefish find food in darkness. *Current Biology* **20**: 1631–1636.

Yoshizawa, M. & Jeffery, W.R. 2011. Evolutionary tuning of an adaptive behavior requires enhancement of the neuromast sensory system. *Communicative & Integrative Biology* **4**: 89–91.

Tables and Figures

Table 1. Descriptive statistics in neuromast numbers of the different neuromast groups. For abbreviations, see Fig. 2.

Neuromast group	Mean	SD	Median	25 percentiles	75 percentiles	Min.	Max.
M	12.87	2.97	13	11	15	4	21
MPrU	12.74	2.86	12	11	14	7	20
Pr-C	8.31	0.69	8	8	8	6	8
Pr	4.75	1.87	5	4	6	0	11
IO	12.35	1.95	12	11	13	8	18
SO-c	8.12	0.55	8	8	8	7	12
POt-c	4.01	0.08	4	4	4	4	5
Ot	9.95	2.77	10	8	11	2	17
PO	5.62	1.65	6	5	6	0	14
DH	12.81	3.32	12	10	15	5	22
Op	4.66	4.50	4	0	8	0	21
ATr-c	3.06	3.24	2	0	6	0	10
ATr	11.81	7.53	12	7	16	0	39

Table 2. Results from the GLMM of the opercular lateral line (OP) and anterior trunk lateral line (ATr). Significant results (after the FDR correction) shown in bold font. Nonsignificant effects (when not being part of a higher level significant interaction) are shown as a result of one-by-one back-substitution to the final model.

	ATr				OP			
Effect	*Ndf*	*Ddf*	*F*	*P*	*Ndf*	*Ddf*	*F*	*P*
Habitat (H)	1	1.975	5.65	0.1423	1	1.975	5.11	0.1537
Predation (P)	**1**	**151**	**17.65**	**<0.0001**	**1**	**163**	**20.71**	**<.0001**
Food (F)	**1**	**151**	**6.41**	**0.0124**	1	162	0.14	0.7085
Sex (S)	1	151	1.18	0.2785	1	162	1.14	0.2863
Habitat × P	**1**	**151**	**15.6**	**0.0001**	**1**	**163**	**8.83**	**0.0034**
Habitat × F	1	151	1.29	0.2575	2	161	1.79	0.1711
Habitat × S	1	151	1.31	0.2546	2	161	0.74	0.4811
P × F	1	151	2.68	0.1037	2	161	0.49	0.6122
P × S	1	151	2.05	0.1539	2	161	1.11	0.3325
F × S	**1**	**151**	**8.19**	**0.0048**	3	160	0.58	0.631
Habitat × P × F	1	151	0.79	0.375	4	159	1.35	0.2532
Habitat × P × S	1	151	0.79	0.7982	4	159	1.06	0.3769
Habitat × F × S	**1**	**151**	**7.44**	**0.0071**	6	157	0.97	0.4468
P× F× S	1	151	2.2	0.1398	6	157	0.72	0.6378
Habitat × P × F × S	**1**	**151**	**9.69**	**0.0022**	12	151	0.96	0.4943

Figure 1. Map of Fennoscandia showing the sampling sites for the parental generations of nine-spined stickleback used in the study.

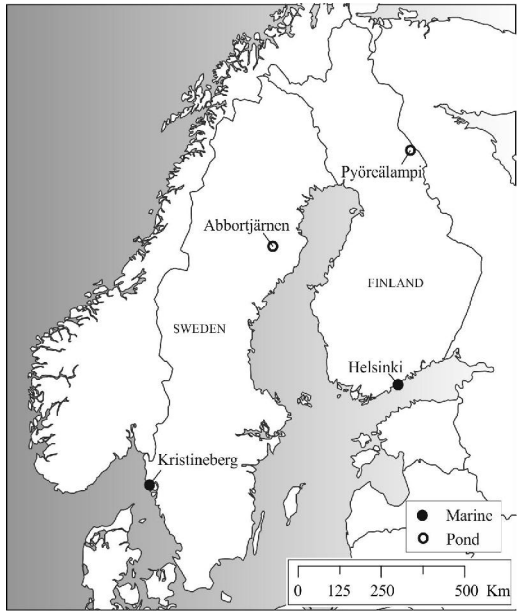


Figure 2. Illustration of the approximate location of analysed groups of neuromasts. Open circles represent canal neuromasts, whereas filled circles represent superficial neuromasts. The abbreviations refer to the following lateral lines (canal neuromasts are indicated with –c suffix): post-otic (POt-c), pre-opercular (Pr-c), supraorbital (SO-c), anterior trunk (ATr-c), upper mandibular preopercular (MPrU), mandibular (M), pre-opercular (Pr), infraorbital (IO), otic (Ot), post-orbital (PO), dorsal head (DH), operculum (OP) and anterior trunk (ATr).

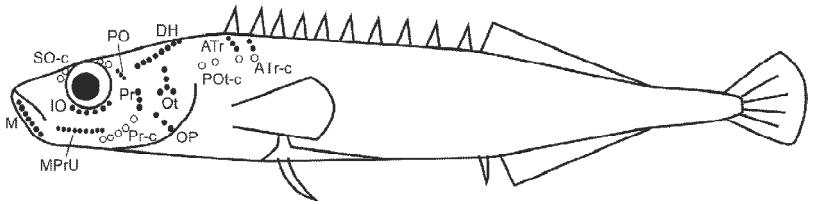
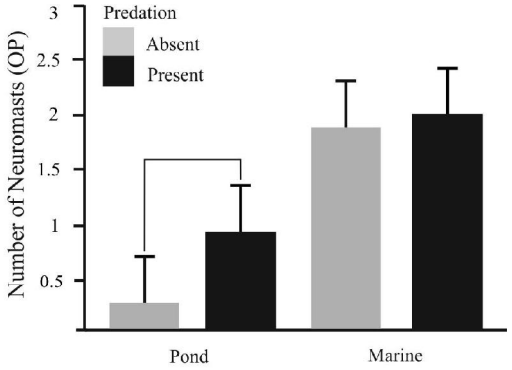
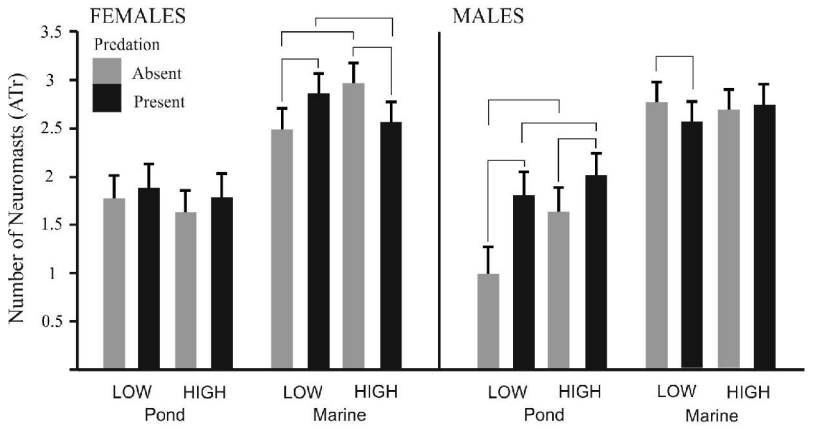


Figure 3. Least square means (± S.E) of neuromast numbers in the opercular (OP) lateral line group in different habitat types and predation risk treatments. Significant pairwise differences within habitat and sex (based on protected Fisher's LSD tests) are marked with connecting lines.



-

Figure 4. Least square means (± S.E) of neuromast numbers in the anterior trunk (ATr) lateral line group in different habitat types, food and predation risk treatments and for both sexes. Significant pairwise differences within habitat and sex (based on protected Fisher's LSD tests) are marked with connecting lines.



SUPPORTING INFORMATION

Additional Supporting Information including tables and figures S1-S4 is available as electronic supplementary file